**Tcf1 and Lef1 orchestrate genomic architecture**

**to supervise mature CD8+ T cell identity and function**

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**T-cell identity is established during thymic development, but how it is maintained in periphery remains unknown. Here we show ablating Tcf1 and Lef1 transcription factors in mature CD8+ T cells aberrantly induces genes in non-T cell lineages. Using high-throughput chromosome-conformation-capture sequencing, we demonstrate that Tcf1/Lef1 are critical for maintaining three-dimensional genome organization at multiple scales in CD8+ T cells. Comprehensive network analyses coupled with genome-wide profiling of chromatin accessibility and Tcf1 occupancy reveal the direct impact of Tcf1/Lef1 on T-cell genome is to promote formation of extensively interconnected hubs through enforcing chromatin interaction and accessibility. The integrative mechanisms utilized by Tcf1/Lef1 underlie activation of T-cell identity genes and repression of non-T lineage genes, conferring fine control of various T-cell functionalities. These findings suggest that Tcf1/Lef1 orchestrate global genome organization and help form intricate chromatin-interacting hubs to facilitate promoter-enhancer/silencer contact, hence providing constant supervision of CD8+ T cell identity and function.**

All immune cells are differentiated from multipotent hematopoietic stem cells (HSCs) following relatively well-defined maturation steps[1](#_ENREF_1). In the differentiation processes, lineage-restricted transcription factors (TFs) are induced and in turn establish identity of a specific cell type[2](#_ENREF_2), [3](#_ENREF_3), [4](#_ENREF_4). While cell identity is thought to be maintained by heritable epigenetic modifications of DNA and histones[5](#_ENREF_5), it is suggested that the lineage-restricted TFs remain necessary to provide constant supervision of cell identity[6](#_ENREF_6). For example, while the Pax5 TF is necessary for committing early B cell progenitors to the B cell lineage[7](#_ENREF_7), deletion of Pax5 in mature B cells causes their dedifferentiation to acquire HSC-like properties and even give rise to T lineage cells[8](#_ENREF_8). Additionally, ablating Foxp3 in immunosuppressive regulatory T cells induces genes characteristic of proinflammatory responses[9](#_ENREF_9). TFs, including lineage-restricted ones, frequently show pervasive occupancy in the genome[6](#_ENREF_6), and their interplay with three-dimensional (3D) genome is well recognized[10](#_ENREF_10). A few TFs, such as Bcl11b, are closely associated with extensive rewiring of 3D genome during lineage differentiation from HSCs[11](#_ENREF_11), [12](#_ENREF_12). It is therefore of considerable interest to investigate whether the 3D genome organization underlies TF-mediated gate-keeping of cell identity. In the case of B cells, the genome architecture is perturbed by Pax5 deletion during the processes of both establishing and maintaining B cell identify[12](#_ENREF_12), [13](#_ENREF_13). However, it remains unclear whether the notion that a lineage-defining TF exerts a genome-organizing role to supervise cell identity could be a generalizable concept in immune cells. In addition, there is a prevalent disconnect between changes in 3D genome and transcriptome thus far, and a causative effect of 3D genome rewiring on transcriptional output remains obscure in a given immune cell type.

CD8+ T lymphocytes are essential for mounting protective cellular immune responses against pathogenic antigens and malignantly transformed cells[14](#_ENREF_14), [15](#_ENREF_15). During early stage of T cell development in the thymus, key TFs including Tcf1, Bcl11b and Gata3 are induced to commit early thymic progenitors to the T-cell lineage[2](#_ENREF_2). These TFs remain in commission to promote production of CD4+ or CD8+ single positive T cells at later stages[16](#_ENREF_16). While Tcf1 and its homologue Lef1 TFs are dispensable for CD8+ lineage choice, they are essential for establishing CD8+ T cell identity by suppressing CD4+ lineage-associated genes[17](#_ENREF_17), [18](#_ENREF_18). It is well established that the *Cd4* gene silencing in CD8+ T cells is epigenetically maintained and heritable through subsequent mitoses[19](#_ENREF_19). It is not known if this is a general rule for all aspects of CD8+ T cell identity after CD8+ T cells complete intrathymic maturation and populate the peripheral lymphoid organs.

Tcf1 and Lef1 are members of the Tcf/Lef subfamily of high-mobility-group (HMG) proteins, containing a single HMG DNA-binding domain with high-degree conservation. The HMG proteins are known to interact with minor groove of the DNA double-helix and thus bend DNA to modulate DNA structure[20](#_ENREF_20). Specifically, Lef1 is shown to bind a minimal TCRa enhancer and induce a sharp bend in DNA *in vitro*; the DNA bending in turn facilitates interaction of Ets and Runx family TFs that bind to sequences flanking the Lef1 site[21](#_ENREF_21), [22](#_ENREF_22). It has been postulated that Tcf1/Lef1 TFs may have a structural role in T cells; however, it remains unknown if such structural role is operating *in vivo* and has functional impact. In this study, we employed multiomics approaches including Hi-C to demonstrate that Tcf1/Lef1 TFs critically regulate global genomic organization in mature CD8+ T cells and provide constant supervision of CD8+ T cell identity and function.

**Results**

**Tcf1 and Lef1 regulate genomic organization on multiple scales in mature CD8+ T cells**

Tcf1 and Lef1 TFs are functionally redundant in establishing CD8+ T cell identity during late stages of thymic development[17](#_ENREF_17). To specifically determine their functions in mature CD8+ T cells, hCD2-Cre, which is most active in mature T cells in the periphery[23](#_ENREF_23), [24](#_ENREF_24), was crossed to *Tcf7* (encoding Tcf1)- and *Lef1*-floxed alleles to generate hCD2-Cre+ *Rosa26*GFP*Tcf7*+/+*Lef1*+/+ and hCD2-Cre+*Rosa26*GFP *Tcf7*FL/FL*Lef1*FL/FL mice (called WT and dKO, respectively). Unlike CD4-Cre-mediated Tcf1/Lef1 deletion, the use of hCD2-Cre did not cause derepression of CD4 coreceptor in dKO mature CD8+ T cells (**Fig. S1a**), validating specific deletion in post-thymic T cells. To directly address the postulated structural role of Tcf1/Lef1 TFs *in vivo*, we performed high-throughput chromosome-conformation-capture sequencing (Hi-C) on WT and dKO naïve CD8+ T cells in two biological replicates (**Fig. S1b**). The Hi-C libraries were reproducible between the replicates (**Fig. S1c**), which were then pooled for most downstream analyses to improve sensitivity (except for identification of differential chromatin loops and interactions).

To capture Tcf1 binding events without relying on a single epitope of Tcf1 protein, recombinant full-length Tcf1 protein was used as immunogen to generate a new Tcf1 antiserum, and its specificity and ability to immunoprecipitate Tcf1 protein were validated (**Fig. S2a–c**). We then used the Tcf1 antiserum for ChIP-seq on WT or Tcf1-deficient CD8+ T cells, which gave strong signal to noise ratio as observed at known Tcf1 target gene loci including *Cd4* and *Tcf7* itself (**Fig. 1a**) (the antiserum will be made available upon request). Using MACS2[25](#_ENREF_25) with stringent criteria (fourfold enrichment, *p* < 10−5 and FDR < 0.05), we identified 19,042 high-confidence Tcf1 binding peaks in WT CD8+ T cells. In motif analysis with HOMER[26](#_ENREF_26), we applied a position-weight matrix to determine the likelihood that a Tcf1 peak resulted from direct binding to the consensus Tcf/Lef motif. This approach provided a quantifiable “motif score” that allowed us to discern direct vs. indirect Tcf1 binding events. 2,866 Tcf1 peaks with a motif score ≥7 were highly enriched in the Tcf/Lef motifs and considered Tcf1 direct binding sites (**Fig. S2d**), while 5,197 Tcf1 peaks with a motif score ≤3 lacked the Tcf/Lef motifs and were consider Tcf1 indirect binding sites (**Fig. S2d**). A comparison of Motif+ Tcf1 peaks with those with intermediate motif scores and Motif– ones in molecular analyses can thus help discern if direct Tcf1 binding is associated with a preferred functional output. For example, while Motif– Tcf1 indirect binding sites were more frequently found in gene promoters (defined as +/– 1 kb region flanking gene transcription start sites, TSS), Motif+ Tcf1 direct binding sites were predominantly localized in regions distal to promoters (gene body and intergenic regions) (**Fig. S2e**). Tcf1 direct binding sites harbored consensus Tcf/Lef motifs as expected, while Tcf1 indirect binding sites were highly enriched with motifs of ETS family TFs (**Fig. S2f**).

On the top hierarchy of genomic organization, AB compartments are recognized as discernible large-scale structures, with A compartment being more transcriptionally active and B compartments being more silent[27](#_ENREF_27). Analysis of the compartment scores in WT CD8+ T cells revealed that Tcf1 binding peaks were highly associated with A compartment (**Fig. 1b**). Comparison between WT and dKO CD8+ T cells showed clearly discernable decrease in scores of compartments harboring multiple Tcf1 peaks upon loss of Tcf1/Lef1 TFs (**Fig. 1c**), suggesting that Tcf1 and Lef1 promote transcriptional activity of compartments in mature CD8+ T cells.

Topological associated domains (TADs) constitute the next level of genomic organization[28](#_ENREF_28). Using the Arrowhead algorithm[29](#_ENREF_29), 1,723 TADs were identified in WT CD8+ T cells, and the TAD boundaries were enriched with CTCF, but not Tcf1 binding peaks (**Fig. S3a**), based on CTCF ChIP-Seq in total T cells[30](#_ENREF_30). Analysis of TAD scores, as a measure for assessing levels of intra-TAD chromatin interactions, revealed that Tcf1 peaks were associated with higher TAD scores in WT CD8+ T cells (**Fig. 1d**). While no apparent changes in TAD boundaries were found between WT and dKO CD8+ T cells, TAD scores were diminished by Tcf1/Lef1 deficiency, and the decrease in TAD scores in dKO CD8+ T cells was more pronounced in TADs with higher density of Tcf1 peaks (**Fig. 1e**). For examples, TADs covering the *Map3k5* gene and *Ccdc33* to *Pml* gene loci showed diminished intra-TAD interactions in dKO CD8+ T cells (**Fig. 1f**).

To determine the impact of Tcf1/Lef1 deficiency on genomic structure on a finer scale, we analyzed chromatin interactions and loops at a 10-kb resolution. By defining interaction scores as a measure for assessing interactions of a given 10-kb bin with the rest of the chromosome, we found that the density of Tcf1 peaks was positively associated with chromatin interaction scores in WT CD8+ T cells (**Fig. S3b**). Whereas the interaction scores for chromatin regions without Tcf1 peaks were similar between WT and dKO CD8+ T cells, those for Tcf1-associated chromatin regions were diminished in dKO CD8+ T cells, and the decrease was more pronounced in regions with higher density of Tcf1 peaks (**Fig. S3c**). To reduce noise intrinsic to Hi-C data, HiCCUPs algorithm[29](#_ENREF_29) was used to determine statistically significant chromatin looping events. In WT CD8+ T cells, we identified 11,490 high-confidence chromatin loops which encompassed enhancer-promoter (EP), promoter-promoter (PP) and enhancer-enhancer (EE) interactions, with the EP loops observed at a higher frequency of ~45%. Tcf1 peaks were highly enriched in all three groups of chromatin loops (**Fig. 1g**, left), and focused analysis of Motif+ Tcf1 peaks showed that Tcf1 direct binding sites were most enriched in EE chromatin loops (**Fig. 1g**, right), consistent with their preferential localization in distal regulatory regions (**Fig. S2e**).

Because chromatin loops rarely work in isolation[31](#_ENREF_31), we examined the chromatin loops from a network perspective using the igraph platform[32](#_ENREF_32) (**Fig. S3d**). Densely inter-connected chromatin loops formed 306 distinct hubs in WT CD8+ T cells, and in these hubs, Tcf1 peaks were more enriched in top hub anchors with the highest connectivity, compared with bottom hub anchors with the lowest connectivity. In contrast, anchors on isolated loops were rarely bound by Tcf1 peaks (**Fig. 1h**). Analysis of differential chromatin loops between WT and dKO CD8+ T cells identified 877 loops that decreased in strength significantly from WT to dKO CD8+ T cells. On a global scale, the strength of chromatin loops was similar between WT and dKO CD8+ T cells (**Fig. 1i**, rightmost column); however, the loops that harbored Tcf1 peaks at both loop anchors exhibited reduced interaction strength in dKO CD8+ T cells, and this tendency was more evident for loops harboring Motif+ Tcf1 peaks (**Fig. 1i**, leftmost two columns). Interestingly, the loops that had Motif+ Tcf1 peak(s) in one anchor also showed weakened interaction in dKO CD8+ T cells (**Fig. 1i**, middle columns). For example, a chromatin loop linking *Rbm45* and *Prkra* gene loci in WT cells, with a Tcf1 peak at the anchor harboring the *Rbm45* TSS, was diminished in strength in dKO CD8+ T cells (**Fig. 1j**). On the other hand, 1,682 chromatin loops were significantly stronger in dKO than in WT CD8+ T cells, suggesting that Tcf1/Lef1 TFs could disengage chromatin interactions, likely through indirect mechanisms (see below). However, Tcf1 peaks, especially the Motif+ Tcf1 peaks, were more frequently associated with WT-specific chromatin loops than with dKO-specific loops, as determined with enrichment analysis (**Fig. 1k**). These observations suggest that Tcf1/Lef1 TFs regulate genomic organization in CD8+ T cells across multiple scales, and promote chromatin interactions at their direct binding sites.

**Tcf1 and Lef1 coordinate chromatin accessibility and super enhancer activity to regulate chromatin interactions in CD8+ T cells**

During thymic developmental stages, Tcf1 is associated with increased chromatin accessibility (ChrAcc) for activation of T-lineage transcriptional program[33](#_ENREF_33), [34](#_ENREF_34), [35](#_ENREF_35). To investigate the impact of Tcf1/Lef1 TFs on mature CD8+ T cells, we used DNase-sequencing to map ChrAcc in WT and dKO CD8+ T cells[11](#_ENREF_11), which formed distinct clusters based on principal component analysis (PCA) (**Fig. S4a**). Among a total of 28,827 ChrAcc sites, 987 sites (3.4%) were more open in WT CD8+ T cells, while 576 sites (2%) showed significantly increase in dKOCD8+ T cells (**Fig. 2a**). Stratifying with Tcf1 peaks revealed that WT-specific ChrAcc sites were predominantly bound by Tcf1 (92%, **Fig. 2a**), and about one third of these Tcf1 peaks were Motif+ Tcf1 direct binding sites (**Fig. 2b**). In contrast, dKO-specific ChrAcc sites were less frequently bound by Tcf1 (41%, **Fig. 2a**), and less than 10% of these Tcf1 peaks were Motif+ (**Fig. 2b**). Chromatin accessibility is more dynamic in enhancers than in promoters[36](#_ENREF_36), and we therefore examined ChrAcc at Tcf1 peaks located in distal regulatory regions (>1 kb from TSS). ChrAcc at Motif+ Tcf1 direct binding sites exhibited significant decrease in dKO compared with WT CD8+ T cells, whereas that at Motif– Tcf1 indirect binding sites were similar between WT and dKO CD8+ T cells, with Tcf1 peaks of intermediate motif cores showing modest changes (**Fig. 2c**). These data indicate that Tcf1/Lef1 TFs, especially at their direct binding locations, have a predominant role in maintaining a chromatin accessible state in mature CD8+ T cells.

We then examined the connection between chromatin accessibility and looping. WT-specific ChrAcc sites were more strongly enriched in WT-specific chromatin loops, whiledKO-specific ChrAcc sites showed stronger enrichment in dKO-specific chromatin loops (**Fig. 2d**), indicating that chromatin looping formation is closely correlated with chromatin open state. In line with this notion, chromatin loops harboring WT-specific ChrAcc sites at single or both anchors showed progressively decreased interaction strength in dKO compared with WT CD8+ T cells (**Fig. 2e**); concordantly, chromatin loops harboring dKO-specific ChrAcc sites at single or both anchors showed progressively increased interaction strength in dKO over WT CD8+ T cells (**Fig. 2e**). Because over 90% WT-specific ChrAcc sites were bound by Tcf1 (**Fig. 2a**), the decreased strength of chromatin loops and the associated decreased ChrAcc sites could be both attributed to direct impact by Tcf1/Lef1 TFs, highlighting their critical roles in coordinating chromatin interaction and accessibility in mature CD8+ T cells.

Super enhancers (SEs) contribute to control of cell identity, and in T lineage cells, *Tcf7* gene locus is regulated by an SE[37](#_ENREF_37), [38](#_ENREF_38). We performed H3K27ac ChIP-seq on WT and dKO CD8+ T cells, which exhibited distinct profiles on PCA (**Fig. S4b**). By use of the ROSE algorithm[38](#_ENREF_38) to stitch and rank H3K27ac-enriched regions called by SICER[39](#_ENREF_39), 1,160 SEs were identified in WT CD8+ T cells, and these SEs were enriched with Tcf1 binding peaks (**Fig. 2f**). By comparing SEs identified in WT and dKO CD8+ T cells with EdgeR at FDR<0.05, we found that 174 and 163 SEs exhibited consistently stronger H3K27ac signals in WT and dKO CD8+ T cells, respectively. Because of the larger scale of SEs where not all individual enhancers exhibited similar changes, these ‘differential’ SEs were hereby referred to as WT- or dKO-prepotent SEs rather than WT- or dKO-specific SEs CD8+ T cells. While Tcf1 peaks were enriched in both WT- and dKO-prepotent SEs, Tcf1 binding events were more enriched in WT-prepotent SEs (**Fig. 2g**). In examining the connection of SEs with chromatin looping, we observed that chromatin loops that overlapped with WT-prepotent SEs at single or both anchors showed progressively decreased interaction strength in dKO compared with WT CD8+ T cells (**Fig. 2h**). On the other hand, chromatin loops that overlapped with dKO-prepotent SEs at both anchors, but not at a single anchor, showed significantly increased interaction strength in dKO over WT CD8+ T cells (**Fig. 2h**). These observations suggest that Tcf1/Lef1 TFs concordantly modulate SE activity and chromatin loop strength in mature CD8+ T cells.

**Tcf1 and Lef1 broadly repress non-T lineage enriched genes in mature CD8+ T cells**

To define the impact of Tcf1/Lef1 deficiency on CD8+ T cell biology, RNA-seq analysis was performed on WT and dKO CD8+ T cells, which showed distinct expression clusters on PCA (**Fig. S4c**). By criteria of ≥ 2-fold expression changes and p < 0.05, we found that 258 genes were downregulated, while 313 genes were upregulated in dKO CD8+ T cells. Functional annotation using DAVID Bioinformatics Resources[40](#_ENREF_40) showed that “immunity”, “adaptive immunity” and “innate immunity” were the top enriched functional clusters in both down- and upregulated genes (**Fig. 3a**), suggesting a close link of Tcf1/Lef1 TFs with immune cell identity-related genes.

To further develop this notion, we retrieved RNA-Seq data from a recent study that characterized the transcriptomes of 86 immune cell populations encompassing lymphoid and myeloid hematopoietic lineages in mice[41](#_ENREF_41). We extracted lineage-enriched genes (LEGs) from seven cell types, which included pan-T cells (including naïve CD8+, conventional naïve CD4+, Treg, and gd-T cells), B cells, pan-NK (including CD27+CD11b+, CD27+CD11b–, and CD27–CD11b+ subsets), conventional DCs (cDCs including CD4+ and CD8+ subsets), plasmacytoid DCs (pDCs), monocytes (including Ly6C+ and Ly6C– subsets), and bone marrow-derived granulocytes (**Fig. 3b**, and **Table S1**). For a LEG, its enrichment in a specific lineage was defined as being expressed at least 5-fold higher than 5 out of 6 other lineages, with an FDR <0.01. This definition was based on the consideration that many genes are expressed in more than one lineages during lineage development, activation, and/or differentiation process in immune responses. We then used gene set enrichment analysis (GSEA) to assess the behavior of the immune LEGs as gene sets without preset threshold. Pan-T cell LEGs were enriched in WT CD8+ cells (**Fig. 3c**); for example, *Ccr7*, encoding the CCR7 chemokine receptor that directs naïve T cell trafficking to secondary lymphoid organ, showed diminished expression in dKO CD8+ T cells (**Fig. S5a**). Surprisingly, all non-T cell LEGs (including B, NK, cDC, pDC, monocytes and granulocytes) were strongly enriched in dKO CD8+ T cells (**Fig. 3d**). These genes include *Cd19*, *Cd79a* and *Cd79b* that encode the Ig-a and Ig-b BCR components, *Ccl5* that augments NK activity[42](#_ENREF_42), *Flt3* that is critical for homeostatic DC division[43](#_ENREF_43), and *Cebpd* that encodes C/EBPd and modulates monocyte differentiation[44](#_ENREF_44) (**Fig. S5a**). These observations suggest that in mature CD8+ T cells, Tcf1/Lef1 TFs are required to provide constant supervision of CD8+ T cell identity, to actively suppress aberrant transcription of non-T lineage-associated genes.

Within the T cell lineage, we previously reported that during thymic development, Tcf1 and Lef1 repress CD4+ lineage-associated genes and effector CD8+ T cell genes in thymic CD8+ T cells[17](#_ENREF_17). To discern if these requirements persist in mature CD8+ T cells in the periphery, we performed clustering analysis within all T cell subsets and found that effector CD8+ T cells had a distinct transcriptome profile from other naive T cell subsets (**Fig. S6a**). Focused analysis of naïve T cells identified naïve CD8+, CD4+, Treg and gdT cell LEGs (**Fig. S6b** and **Table S2**). GSEA showed that not only effector CD8+ but also Treg and gdT cell LEGs were enriched in dKO CD8+ T cells (**Fig. S6c**). For example, *Gzmb* (encoding granzyme B, a key cytotoxic molecule), *Prdm1* (encoding the Blimp1 TF that promote effector T cell differentiation), *Foxp3* (the lineage-defining TF of Treg cells), and *Nrp1* (encoding a Treg surface marker Neurophilin-1)[45](#_ENREF_45) were highly expressed in dKO CD8+ T cells (**Fig. S5b**). In contrast, both naïve CD8+ and naïve CD4+ T cell LEGs, which were more limited in numbers, showed modest enrichment in WT CD8+ T cells (**Fig. S6d**). This focused analysis on T-lineage cells indicate that Tcf1/Lef1 TFs remained necessary to suppress aberrant expression of cytotoxic effector-associated and Treg lineage enriched genes. It should be noted, however, the increased transcripts of non-T or non-cytotoxic lineage genes in dKO CD8+ T cells do not mean that Tcf1/Lef1-deficient CD8+ T cells were transformed into other cell types such as B cells, DCs or Treg cells, because they retained the capacity of inducing cytotoxic cytokines upon activation (detailed below).

**Tcf1 and Lef1 utilize chromatin accessible sites as transcription enhancers or silencers**.

To determine how the impact of Tcf1/Lef1 TFs on chromatin and genomic organization is connected to transcriptional output in mature CD8+ T cells, we took a gene-centric approach by pooling DEGs defined by the preset thresholds and differentially expressed lineage enriched genes (DLEGs) defined by leading edges based on GSEA (**Table S3**). The DEG+DLEG approach identified 847 up- and 283 down-regulated genes in dKOCD8+ T cells, which were defined as Tcf1/Lef1-repressed and -activated genes, respectively. To link differential ChrAcc with these genes, we adopted the following association rules to include ChrAcc sites 1) at the promoter region, 2) within gene body and 50 kb upstream of TSS, and 3) connected to gene promoters *via* chromatin loops (not limited to differential loops between WT and dKO CD8+ T cells). One hundred and seventy-five Tcf1/Lef1-repressed genes were linked to 220 differential (Diff) ChrAcc sites, with 60% showing increased ChrAcc and the rest showing decreased ChrAcc (clusters 1 and 2 in **Fig. 4a**, respectively; **Table S4**). Thirteen Diff ChrAcc sites in cluster 1 were at the promoters including *Gzma* and *Gzmb* (effector CD8+ LEGs, **Fig. 4b**), and others were at distal regulatory regions, such as introns of *Blk* (a B cell LEG) and *Ctla4* (a Treg cell LEG) (**Fig. 4c**). Counter-intuitively, Cluster 2 Diff ChrAcc sites showed direct linkage of decreased ChrAcc with increased gene expression. These sites were found at downstream of *Fasl* (an effector CD8+ LEG), introns of *Cd40lg* (a CD4+ LEG), *Pax5* and *Syk* (B cell LEGs) (**Fig. 4d**), upstream of *Gzmb* (**Fig. 4b**) and *Prdm1* (effector CD8+ LEGs, **Fig. 5b**). This Diff ChrAcc cluster showing changes discordant with expression of linked genes may function as transcriptional silencers/repressors (see below).

On the other hand, 80 Tcf1/Lef1-activated genes were linked to 113 Diff ChrAcc sites, with most of these sites showing decrease in ChrAcc in dKO CD8+ T cells (C4 in **Fig. 4a**; **Table S4**). Fourteen genes including *Gria3* (a naïve CD8+ LEG, encoding glutamate receptor C) and *Pou2af1* (an OCT TF-associated transcriptional coactivator) exhibited decreased ChrAcc at their promoters (**Fig. 4e**). Other genes had decreased ChrAcc at distal regulatory regions, such as two upstream sites at *Tubb3* (tubulin b3) and an intron site in *Bach2* (**Fig. 4f**). The minor C3 cluster showed increased ChrAcc in dKO CD8+ T cells, discordant with gene expression and might have repressive regulatory functions.

Parsing Diff ChrAcc clusters with Tcf1 occupancy revealed that Tcf1 peaks were predominantly enriched in C2 and C4 clusters where ChrAcc was diminished in dKO CD8+ T cells (**Fig. 4a**). Of note, high-confidence Tcf1 peak(s) were found to overlap with all 79 ChrAcc sites in C2 cluster and 98 out of 108 sites in C4 cluster, and these Tcf1-bound ChrAcc sites exhibited a range of motif cores for Tcf/Lef motif (**Fig. 4a**,right panels). This observation suggests that Tcf1/Lef1 TFs have a direct role in keeping chromatin at an accessible state in mature CD8+ T cells. In contrast, ChrAcc sites in C1 cluster that were increased in dKO CD8+ T cells showed sparse overlap with high-confidence Tcf1 peaks, indicative of indirect mechanisms by which Tcf1/Lef1 TFs restrain ChrAcc at these locations. Because C2 and C4 ChrAcc clusters were associated with opposite gene expression patterns in dKO CD8+ T cells, we examined relative enrichment of Tcf1 direct binding sites within the two clusters but found only modest bias of Motif+ Tcf1 peaks in the C2 cluster (**Fig. 4g**). We next checked H3K27ac state in the ChrAcc clusters. Both C1 and C4 ChrAcc sites showed concordant changes in H3K27ac signals, with C4 sites showing stronger H3K27ac in WT CD8+ T cells than in dKO cells (**Fig. 5a**). Specifically, the ChrAcc sites observed in *Tubb3* upstream and *Bach2* intron regions were associated with strong H3K27ac signals in WT CD8+ T cells, and both ChrAcc and H3K27ac signals were markedly reduced in dKO CD8+ T cells (**Fig. 4f**). These observations are consistent with the notion that C4 ChrAcc sites are Tcf1/Lef1-dependent transcriptional enhancers to activate the expression of linked genes.

In contrast, H3K27ac signals in C2 ChrAcc clusters were substantially weaker than those in C4 clusters in WT CD8+ T cells, but were similar between WT and dKO CD8+ T cells (**Fig. 5a**, middle panel). This distinct pattern was consistently observed at the ChrAcc sites in introns of *Cd40lg*, *Pax5*, and *Syk* (**Fig. 4d**) and upstream of *Prdm1* (**Fig. 5b**). The lack of H3K27ac at C2 ChrAcc sites suggests that these chromatin accessible sites do not function as enhancers, but may instead act as Tcf1/Lef1-dependent transcriptional silencers/repressors. To further investigate this notion, we focused on the *Prdm1* gene, which was upregulated in dKO CD8+ T cells. The *Prdm1* locus contained at least two high-confidence Tcf1 peaks, one at the –24 kb upstream of *Prdm1* TSS and the other in intron 3, and both Tcf1 binding sites exhibited strong chromatin accessibility in WT CD8+ T cells (**Fig. 5b**). The *Prdm1* –24 kb ChrAcc site was in C2 and became less accessible in dKOCD8+ T cells, and H3K27ac signals at this site were at the background level in both WT and dKO CD8+ T cells (**Fig. 5b**). In contrast, the *Prdm1* intron3 ChrAcc site in WT CD8+ T cells remained similarly accessible in dKO CD8+ T cells. This site showed strong H3K27ac signals in WT CD8+ T cells, which were modestly elevated, if any, in dKO CD8+ T cells (**Fig. 5b**). We have previous targeted each site in germline using CRISPR/Cas9 approach and generated *Prdm1*(–24kb)M and *Prdm1*(Int3)M alleles separately[46](#_ENREF_46), [47](#_ENREF_47). Interestingly, *Prdm1*(–24kb)M/M naïve CD8+ T cells showed elevated *Prdm1* expression than WT cells (**Fig. 5c**), supporting the notion that the *Prdm1* –24kb site is a Tcf1/Lef1-dependent transcriptional repressor element. In contrast, *Prdm1* expression was reduced in *Prdm1*(Int3)M/M compared with WT CD8+ T cells (**Fig. 5c**), suggesting the *Prdm1* intron 3 site functions as a Tcf1/Lef1-independent transcriptional enhancer to support basal *Prdm1* transcription in naïve CD8+ T cells.

**Tcf1 and Lef1 modulate super enhancer activity for target gene regulation**

We next examined the contribution of Tcf1/Lef1-modulated SEs to target gene regulation. Parsing differential SEs with Tcf1/Lef1 target genes revealed that changes in SE signal strength were mostly concordant with those in expression of SE-associated genes (**Fig. 5d**, quadrant *iii*; **S7a**, left; **Table S5**). Forty-seven Tcf1/Lef1-activated genes were associated with 35 WT-prepotent SEs. For example, *Ccr7* was within an SE that had stronger H3K27ac signals in WT than dKO CD8+ T cells (**Fig. 5e**). Another pan-T cell LEG *Inpp4b* encompasses 785 kb, and its gene body harbored an SE with stronger H3K27ac in WT CD8+ T cells (**Fig. 5e**). Both genes contained multiple Tcf1 peaks within the gene body, and *Ccr7* was associated with several Tcf1 peaks in its flanking genomic regions. It is of note that only one Tcf1 peak in *Ccr7* and two Tcf1 peaks in *Inpp4b* SEs overlapped with decreased ChrAcc in dKO CD8+ T cells; in contrast, the SE regions extensively bound by Tcf1 showed broadly stronger H3K27ac in WT over dKO CD8+ T cells (**Fig. 5e**). These observations suggest that Tcf1/Lef1 TFs directly modulate SE activity to activate their target gene transcription, in addition to maintaining chromatin open state at select regulatory elements.

On the flip side, 78 Tcf1/Lef1-repressed genes were associated with 62 dKO-prepotent SEs (**Fig. 5d**, quadrant *i*; **S7a**, right; **Table S5**). For example, *Cish* and *Cx3cr1* (effector CD8+ and Treg LEGs, respectively) were linked to SEs with elevated H3K27ac in dKO CD8+ T cells (**Fig. 5f**), and both SEs harbored multiple Tcf1 peaks in WT CD8+ T cells. While *Cish*-linked SE did not show ChrAcc changes, *Cx3cr1*-linked SE contained two increased ChrAcc sites in dKO CD8+ T cells, which did not overlap with Tcf1 peaks (**Fig. 5f**), consistent with a pattern observed in ChrAcc C1 cluster (**Fig. 4a**). These observations suggest that Tcf1/Lef1 TFs are directly involved in dampening the activity of a group of SEs in mature CD8+ T cells, but largely through indirect mechanisms.

**Tcf1 and Lef1 modulate chromatin interactions for target gene regulation**

Whereas Tcf1/Lef1 deficiency affected the strength of select chromatin loops defined by stringent statistical significance, such as the one observed between *Rbm45* and *Prkra* gene loci (**Fig. 1j**), a more frequently observed impact was diminished chromatin interactions within a TAD or sub-TAD, as observed in the *Map3k5* locus (**Fig. 1f**, left panel). To systematically identify such regions and assess their effect on the expression of target genes, we adopted the approach of network analysis. We first extracted all interactions with the same directional changes between WT and dKO CD8+ T cells, and then identified 3D chromatin interaction clusters based on connectivity among bins anchoring these interactions (**Fig. 6a**). The 3D clusters were then projected onto one-dimensional genomic regions, followed by integrative comparative analysis of numbers, strength and statistical significance of chromatin interactions among the regions. This approach identified 221 WT-specific and 194 dKO-specific chromatin interaction hubs (**Fig. 6a**). For example, in the interaction network of a chromosome 10 segment, a cluster harboring the *Myb* gene formed a WT-specific hub, where chromatin interactions were stronger in WT than dKO CD8+ T cells (**Fig. 6b**). Similarly, in the network of a chromosome 11 segment, the cluster harboring the *Ccl3*, *Ccl4*, *Ccl9*, and *Ccl5* gene loci formed a dKO-specific hub (**Fig. 6c**). Notably, both WT- and dKO-specific hubs were enriched with Tcf1 peaks, and the Tcf1 peak enrichment was substantially higher in WT-specific hubs (**Fig. 6d**), indicating a predominant role of Tcf1/Lef1 TFs in promoting chromatin interactions and hub formation.

To connect the hubs to gene expression changes, hubs containing at least one DEG+DLEG promoter were identified and plotted against gene expression changes (**Fig. 6e**, **Table S6**). This approach revealed strong association of WT-specific hubs with Tcf1/Lef1-activated genes including the Myb TF and IL-6 receptor a chain (encoded by *Il6ra*) along with several pan-T cell LEGs such as *Cpm*, *Dapl1*, *Gabrr2*, *Inpp4b*, *Prrg1* and *Rgs11* (**Fig. 6e**, quadrant *iii*). Consistent with localization of the *Myb* gene locus within a WT-specific hub (**Fig. 6b**), the *Myb* promoter showed extensive interactions with its upstream regions that spanned over 100 kb, and the chromatin interactions were greatly diminished in dKO compared with WT CD8+ T cells (**Fig. 6f**). In addition, through hub analysis, the *Myb* promoter was linked to three ChrAcc sites showing significant reduction in dKO CD8+ T cells (**Fig. 6b,g**), complementing the empirical linking rule because these sites were >50 kb upstream of Myb TSS. Furthermore, the *Myb* locus was in a WT-prepotent SE (**Fig. 5e,6g**). Similarly, the *Inpp4b* (a pan-T LEG) locus was in a WT-specific hub (**Fig. S7b**), showing decreased intronic interactions in dKO CD8+ T cells (**Fig. S7c**). The *Inpp4b* promoter was linked to two ChrAcc sites showing reduction in dKO CD8+ T cells (**Fig. S7b, 5e**), and the locus harbored a WT-prepotent SE (**Fig. 5d, 5e**). These analyses revealed that Tcf1/Lef1 TFs deployed mechanisms at multiple levels to ensure positive regulation of key targets in mature CD8+ T cells to enforce T cell identity.

It is of note that a fraction of genes associated with WT-specific hubs showed elevated expression, such as *Gata3*, *Havcr2*, *Il12rb2*,*Islr*, *Ldlrad3* and *Syk* (**Fig. 6e**, quadrant *ii*), suggesting that Tcf1/Lef1-mediated chromatin interactions could exert repressive function on transcription. In line with this notion, *Havcr2*, *Il12rb2*,*Ldlrad3* and *Syk* genes were linked with Diff ChrAcc Cluster 2 (*i.e*., decreased ChrAcc sites coupled with increased gene expression in dKO CD8+ T cells, **Fig. 4a**; one example of such sites can be found in the *Syk* intron, **Fig. 4d**), where the ChrAcc sites may function as transcriptional repressors. As a highlight of this point, the *Ldlrad3* (a pDC or monocyte LEG) gene locus was in a WT-specific hub formed on a chromosome 2 segment (**Fig. 7a**), and its gene body and downstream region showed decreased chromatin interactions in dKO CD8+ T cells (**Fig. 7b**). Additionally, the *Ldlrad3* hub contained a Cluster 2 ChrAcc site in the proximity of *Ldlrad3* promoter (**Fig. 7a**), which was found at 2.4 kb upstream of *Ldlrad3* TSS (**Fig. 7c**). These data underscore the integrative power of network analysis, suggesting that Tcf1/Lef1 TFs could promote chromatin accessibility and interactions to achieve repression of lineage-inappropriate genes in mature CD8+ T cells.

On the other hand, dKO-specific interaction hubs were more frequently associated with Tcf1/Lef1-repressed genes, which include many non-T cell LEGs such as *Igf1r* (granulocyte), *Xylt1* (monocyte), *Rab38* (cDC), *Prkcg* and *Mrgpre* (pDC), *Rab30* (B cell), and *Nkg7* (NK cell LEG) (**Fig. 6e**, quadrant *i*). Of particular interest was a dKO-specific hub that contained multiple CCL genes including *Ccl3*, *Ccl4*, *Ccl6*, and *Ccl5* (defined as NK, gdT and effector CD8+ T cell LEGs) (**Fig. 6c**). In this hub, a >200 kb region exhibited extensive increase in chromatin interactions in dKO CD8+ T cells, including regions with direct contact with the *Ccl* gene promoters (**Fig. 7d**). In addition, several dKO-specific ChrAcc sites were linked to the *Ccl* genes, including one at the *Ccl4* promoter and several upstream of *Ccl5* (**Fig. 7e**). The *Ccl* genes were all associated with a single SE with elevated H3K27ac in dKO CD8+ T cells (**Fig. 7e**). The *Ccl*-linked hub contained multiple Tcf1 peaks; nonetheless, none of these peaks contained Tcf/Lef consensus motifs. These observations suggest that Tcf1/Lef1 TFs is directly involved, but maybe through indirect mechanisms, in restraining chromatin accessibility and/or disengaging chromatin interactions to repress expression of CD8+ lineage-inappropriate genes.

**Tcf1 and Lef1 control multiple aspects of CD8+ T cell functionality**

Detailed molecular analyses above identified novel Tcf1/Lef1 target genes in mature CD8+ T cells and the underlying regulatory mechanisms utilized by the TFs. To determine how Tcf1/Lef1 control CD8+ T cell biology through their downstream transcription program, we validated protein expression changes in dKO CD8+ T cells for select target genes and then investigated their functional link with T cell biology. Tcf1/Lef1 deficiency diminished SE activity at the *Ccr7* gene locus (**Fig. 5e**), and dKO CD8+ T cells exhibited greatly reduced CCR7 expression (**Fig. 8a**). CCR7 is essential for migration of mature T cells to secondary lymphoid organs; indeed, when CD45.1+ WT and CD45.2+GFP+ dKO CD8+ T cells were adoptively transferred into a new host in 1:1 mixture, the dKO cells showed impaired capacity of homing to lymph nodes (**Fig. 8b**). dKO CD8+ T cells showed reduced Eomes transcripts (**Fig. S5b**) as well as protein (**Fig. 8c**). Consistent with an important role of Eomes in generation and persistence of memory CD8+ T cells elicited by acute infection[48](#_ENREF_48), [49](#_ENREF_49), the CD44hiCD122+ memory-phenotype CD8+ T cells were substantially diminished in uninfected dKO mice (**Fig. 8d**). dKO CD8+ T cells exhibited an enrichment of cytotoxic genes associated with effector CD8+ T cells (**Fig. S6c**), resulting from a multitude of mechanistic actions by Tcf1/Lef1, including regulating a silencer at *Prdm1* locus (**Fig 5b, c**), ChrAcc at *Gzmb* locus (**Fig 4b**), SE and chromatin interaction at a cluster of *Ccl* gene loci (**Fig 7d,e**). Indeed, the basal expression of granzyme B was elevated in dKO CD8+ T cells (**Fig. S8a**), and CCL5 production showed pronounced increase in stimulated dKO CD8+ T cells (**Fig. S8b**). After confirming that dKO CD8+ T cells were not more prone to apoptosis than WT cells after isolated *ex vivo* (**Fig. S8c**), we stimulated the cells with titrating amounts of anti-CD3 and anti-CD28 using an *in vitro* culture system. Both WT and dKO CD8+ T cells showed similar capacity of producing IFN-g and Granzyme B at all doses after 72-hr stimulation (**Fig. S8d**). These observations suggest that the increased expression of effector genes in dKO CD8+ T cells was not translated into lower threshold of activation.

Owing to Tcf1/Lef1-mediated concerted regulation of chromatin interaction, SE activity and ChrAcc at the *Myb* locus (**Fig. 6**), dKO CD8+ T cells showed potent downregulation of *Myb* transcripts (**Fig. S5b**) and MYB protein (**Fig. 8e**). In addition, Treg lineage genes, including *Foxp3* and *Nrp1*, were enriched in dKO CD8+ T cells (**Fig. S6c**), and both FOXP3 and NRP1 proteins showed elevated expression in dKO CD8+ T cells (**Fig. 8f**). MYB is necessary for effector CD8+ T cell expansion in response to viral infection[50](#_ENREF_50), and FOXP3 has broad transcription-suppressive functions that are even extrapolatable to nonlymphoid cells[51](#_ENREF_51). To further test how these transcriptomic and phenotypic changes due to Tcf1/Lef1 deficiency affected CD8+ T cell-mediated immune response *in vivo*, we crossed P14 TCR transgene, which encodes a TCR specific for the LCMV GP33 epitope, onto the dKO background. The resulting P14 CD8+ T cells were adoptively transferred into CD45.1+ congenic recipients, followed by LCMV infection (**Fig. 8g**). By monitoring dilution of cell trace violet (CTV) during the initial 60 hrs post-infection, we found that WT P14 cells exhibited rapid induction of CD25 and active cell division, with most of cells at 3-4 divisions by 48hrs and at 5-6 divisions by 60 hrs (**Fig. 8h–j**). dKO P14 cells were activated, but showed slower rate of cell division at these early time points, with most of cells at the 2nd division by 48hrs and at the 4th division by 60 hrs (**Fig. 8h–j**). The level of CD25 induction was also modestly reduced in dKO cells (**Fig. 8h**,**j**). Consistent with the changes at these early timepoints, on day 8 post-infection when CD8+ T cells reached peak response, the expansion of dKO effector P14 cells was reduced by 2/3 compared with WT cells (**Fig. 8k**). In addition, dKO effector cells showed moderately diminished polyfunctionality in producing IFN-g together with TNF and IL-2 (**Fig. S8e,f**). Collectively, these data demonstrated that ablating Tcf1/Lef1 in naïve CD8+ T cells compromised their functions in multiple aspects at the homeostatic state and in response to pathogen challenge.

**Discussion**

T cell identity is established during thymic development, under the direction of key TFs including Tcf1. Maintenance of T cell identity, in at least some aspects, such as *Cd4* gene silencing, is thought to be autonomous through epigenetic mechanisms[19](#_ENREF_19). In this study, specific ablation of Tcf1 and Lef1 in mature CD8+ T cells resulted in not only diminished expression of T cell lineage enriched genes, but also aberrantly upregulated genes that were enriched in non-T cell lineages including B cells, DCs and monocytes. In addition, Tcf1/Lef1 TFs remain necessary to maintain CD8+ T cells at their naïve state before encountering cognate antigens by repressing cytotoxic genes induced in effector CD8+ T cells. These data demonstrate that CD8+ T cells require constant supervision of their unique identity and naïve state, and Tcf1/Lef1 TFs have essential roles in these critical processes.

In line with a general feature of HMG family proteins, Tcf1/Lef1 TFs have been shown to bend DNA *in vitro* and postulated to have direct impact on DNA structures in the 3D space[21](#_ENREF_21), [22](#_ENREF_22). Comprehensive analysis of Hi-C profiles of WT and Tcf1/Lef1-deficient CD8+ T cells revealed that Tcf1/Lef1 TFs modulate genomic organization on multiple scales including A/B compartments, TADs, hubs and focal chromatin looping, with stronger impact observed at genomic regions with higher density of Tcf1 binding peaks, especially those with direct Tcf1 binding. The direct impact on focal chromatin loops by Tcf1/Lef1 TFs is in line with the punctate nature of Tcf1 binding peaks on the genome. In addition to enriched Tcf1 occupancy at anchors of chromatin loops, extensive Tcf1 binding peaks were found within TADs. Our integrative network analysis revealed quantifiable impact of Tcf1/Lef1 TFs on chromatin interactions, demonstrating wide-spread reduction in contact frequency in Tcf1/Lef1-deficient CD8+ T cells within chromatin interaction hubs. Thus, Tcf1/Lef1 TFs help form highly connected contact matrices to facilitate interactions among neighboring regulatory regions, consistent with their DNA-bending capacity. This observation is compatible with a trending concept that weak but multivalent interactions among macromolecules, such as TFs and their cofactors, form phase-separated condensates for highly coordinated transcriptional control[52](#_ENREF_52), [53](#_ENREF_53).

Complementing the larger scale of Hi-C data, systemic mapping of chromatin accessibility and super enhancer activity uncovered their coordinated actions with chromatin looping in CD8+ T cells. Tcf1 is considered a ‘pioneer’ factor that establishes ChrAcc landscape during T cell development[29](#_ENREF_29), [30](#_ENREF_30), [31](#_ENREF_31). In mature CD8+ T cells, loss of Tcf1 and Lef1 resulted in substantial changes in ChrAcc. Through detailed motif analysis of Tcf1 binding peaks, we found that Tcf1 direct binding sites, where Tcf1 has direct contact with DNA elements, overlapped extensively with ChrAcc sites showing reduced accessibility in Tcf1/Lef1-deficient CD8+ T cells. This finding indicates that the immediate, direct impact by Tcf1/Lef1 TFs on the chromatin is to maintain the ChrAcc at an open state in CD8+ T cells. Importantly, changes in ChrAcc state and super enhancer activity are concordant with the strength of chromatin loops and interactions, suggesting that Tcf1/Lef1 TFs function as key orchestrators of chromatin open state at cis-element levels, super enhancer activity on a larger scale, and genomic organization in 3D space.

As frequently demonstrated, global changes in chromatin state and genomic organization are not always directly consequential in altering transcriptional output. We took a gene-centric approach, *i.e.*, focusing on DEGs and differentially expressed immune cell lineage-enriched genes, which proved to be mechanistically revealing. At Motif+ Tcf1 direct binding sites, Tcf1/Lef1 TFs maintain chromatin at open state and promote chromatin interactions. It is not surprising that these regulatory effects resulted in positive regulation of their downstream target genes including key TFs such as *Myb*. However, the ChrAcc and/or chromatin interactions supported by Tcf1/Lef1 TFs also showed repressive effects depending on the gene loci, as exemplified at the Tcf1-bound, –24 kb element upstream of the *Prdm1* gene. Notably, this upstream *Prdm1* element lacked H3K27ac modification, in key contrast to an active enhancer in *Prdm1* intron 3. These observations suggest the versatility of Tcf1/Lef1 TFs in implementing gene regulation. They directly determine chromatin state and/or interactions, and cooperate with cofactors such as histone modifiers/readers in specific gene context, to achieve an output of either transcriptional activation or repression.

As evident in our systematic molecular analyses, Tcf1 binding events are associated with distinct regulatory effects, such as promoting or disengaging chromatin interaction, increasing or reducing chromatin accessibility, and activating or repressing target gene expression, depending on the gene context. By use of position-weight matrix in motif analysis, we made the distinction between Tcf1 direct vs. indirect binding events and assessed their relative contribution to each regulatory mechanism. At its direct binding sites, Tcf1 exhibited clearly distinguishable preference for promoting chromatin interactions and maintaining chromatin at an open status. Such distinction predicts likelihood of a preferred functional outcome, but should be interpreted in absolute terms. Besides the direct, positive impact by Tcf1/Lef1 TFs on chromatin, we noted that ablation of Tcf1 and Lef1 in mature CD8+ T cells resulted in increased ChrAcc, elevated super enhancer activity, and aberrantly enhanced chromatin interactions at distinct gene loci and/or genomic regions. These changes were associated with abnormal induction of lineage-inappropriate, or effector-associated genes. Whereas scores of Tcf1 peaks could be found in super enhancers, chromatin loop anchors and interaction hubs because of their large sizes, the relative enrichment of Tcf1 binding was substantially lower in these large structures specific to Tcf1/Lef1-deficient CD8+ T cells than in those specific to WT cells. On a finer scale, ChrAcc sites specific to Tcf1/Lef1-deficient CD8+ T cells rarely overlapped with strong Tcf1 binding peaks, and the overlapping Tcf1 peaks rarely contained Tcf/Lef motifs. Therefore, Tcf1/Lef1 TFs could be ‘directly’ involved, but through ‘indirect’ mechanisms, in the repressive roles such as constraining ChrAcc and disengaging chromatin interactions. For example, due to the lack of Tcf/Lef motifs at those Tcf1 peaks, Tcf1 is likely ‘indirectly’ recruited by other TFs that have direct contact with DNA, to serve as a key component in protein complexes exerting repressive roles. In this regard, we previously demonstrated that Tcf1 and Lef1 have intrinsic histone deacetylase activity[17](#_ENREF_17), and in immunization-elicited follicular helper T cells, Tcf1 binds to the *Ctla4* gene locus indirectly to restrain its ChrAcc[54](#_ENREF_54). Other possible mechanisms may involve aberrantly induced proteins and/or increased availability of Tcf1/Lef1 cofactors in Tcf1/Lef1-deficient cells, which in turn promote ChrAcc or chromatin interactions. These possibilities warrant detailed molecular dissection in future investigations.

It should be noted that resolution remains a major limiting factor for Hi-C-based analysis of enhancer-promoter linkage, especially for short-range interactions[12](#_ENREF_12), [13](#_ENREF_13). In this study, we reached 10-kb resolution, which remained lower than what was achieved for DNase-seq, H3K27ac and Tcf1 ChIP-seq. As a result, many known Tcf1/Lef1 downstream genes did not register as being regulated through the structural mechanism. For example, the *Prdm1* gene encompasses a 20.4 kb genomic region, and the upstream silencer, identified in this work based on Tcf1/Lef1-dependent ChrAcc changes, was 24 kb from the *Prdm1* TSS. Resolution at a finer scale is needed to definitively resolve the short-range interactions among *Prdm1* promoter, its intronic enhancer and upstream silencer. Nonetheless, our analysis of super enhancers and genomic architecture on a larger scale did identify novel Tcf1/Lef1 target genes with profound implications in CD8+ T cell biology. These included Tcf1/Lef1-mediated positive regulation of *Ccr7* and Myb, which determined the homing and proliferative capacity of CD8+ T cells, respectively. In fact, the diminished proliferation and poly-cytokine production capability in Tcf1/Lef1-deficient effector CD8+ T cells were a phenocopy of defects observed in Myb-ablated CD8+ T cells[50](#_ENREF_50).

In summary, this study demonstrates that Tcf1/Lef1 TFs directly regulate global genomic organization on multiple scales in mature CD8+ T cells. By adapting network analysis, our systematic approaches enhance integration of Hi-C and multiomics data and uncover concordant modulation of chromatin accessibility, super enhancer activity, chromatin looping and chromatin-interaction hubs by Tcf1/Lef1 TFs. These approaches help establish direct linkage of these multifaceted mechanisms to transcriptional output at immune cell lineage enriched genes, revealing a critical function of Tcf1/Lef1 TFs in supervising mature CD8+ T cell identity to prevent lineage confusion and protecting critical CD8+ T cell functions. Our findings also provide critical insights into the interplay between TFs and 3D genome architecture in general, highlighting the integrative nature of TFs’ actions on transcriptional regulation.

**Accession numbers**

The Hi-C, DNase-seq, RNA-seq, Tcf1 and H3K27ac ChIP-seq data are deposited at the Gene Expression Omnibus under accession number GSE164713.

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**Author contributions**

Q. S. performed the experiments, with assistance from X. C. and K. G.; X. L. analyzed all the high throughput sequencing data, with assistance from S. Z and Z. Z.; W.P. and H.H.X. conceived the project, supervised the study, and wrote the paper. **References**

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**Figure Legends**

**Figure 1**. **Tcf1 and Lef1 regulate global genomic organization in mature CD8+ T cells**.

**a**. Tcf1 ChIP-seq tracks at the *Tcf7* and *Cd4* gene loci in WT and *Tcf7*–/– CD8+ T cells, with gene structure and transcription orientation displayed on top of each panel.

**b**. Tcf1 binding peaks are enriched in A compartments in WT CD8+ T cells. Compartment bins were allocated into different groups based on the density of Tcf1 peaks within each bin, and the A/B compartment score was determined using the Eigenvector function in the Juicer package. Red line in the middle of boxplot denotes median, box denotes interquartile range (IQR), and whiskers denote the most extreme data points that are no more than 1.5 × IQR from the edge of the box. The blue dotted line denotes compartment score at 0. The statistical significance was calculated using one-sided Mann-Whitney U test. This annotation applies to all boxplots in this work.

**c**. Tcf1/Lef1 deficiency diminishes compartment scores in Tcf1-bound compartments. Differential A/B compartment scores were determined between dKO and WT ­CD8+ T cells, and then distributed into different groups based on Tcf1 peak density. The blue dotted line denotes a non-differential score.

**d.** Tcf1 peaks are enriched in TADs with higher scores. TADs were identified using Arrowhead and allocated into different groups based on Tcf1 peak numbers per 100 kb in WT CD8+ T cells. Shown is the distribution of TAD scores in each group.

**e**. Tcf1/Lef1 deficiency diminishes TAD scores. Differential TAD scores were determined between dKO and WT ­CD8+ T cells, and then distributed into different groups based on Tcf1 peak numbers per 100 kb.

**f**. Select differential TADs detected at the *Map3k5* (left) and *Ccdc33* to *Pml* gene loci (right). Diamond graphs showing chromatin interactions in WT (top) and dKO CD8+ T cells (bottom) were extracted from WashU epigenome browser, with gene structures and Tcf1 ChIP-seq tracks in the middle. Genomic and color scales are displayed on the top.

**g**. Tcf1 peaks are enriched in anchors of chromatin loops. Chromatin loops are called using HiCCUPS and allocated into different groups according to anchor annotation in promoters (P) or enhancers (E). The observed numbers of all (left) and Motif+ (right) Tcf1 peaks per loop were determined for each group, and the expected numbers were based on genome average.

**h**.Tcf1 peaks are enriched in the hubs of the chromatin loop network. From the network of chromatin loops in WT CD8+ T cells, 306 hubs were identified as highly inter-connected clusters of interactions. Anchors of hubs were ranked according to interaction frequency, and the top (No. 1) and bottom (last) anchors from each hub were collected and analyzed for Tcf1 peak enrichment. Anchors of the isolated loops were analyzed as a control.

**i**.Tcf1/Lef1 deficiency diminishes the strength of Tcf1-associated chromatin loops. Chromatin loops identified in WT and dKO­ CD8+ T cells were allocated into different groups based on the presence of total and Motif+ Tcf1 peaks at a single or both loop anchors, and changes in loop strength were determined in each group. The blue dotted line denotes no change in loop strength.

**j**. Select differential chromatin loop at the *Rbm45* to *Prkra* gene loci, as denoted by circles.

**k**.Tcf1 peaks are enriched in anchors of WT-specific chromatin loops. Differential chromatin loops were called using EdgeR, and the frequency of all (left) and Motif+ Tcf1 peaks (right) at anchors of WT- or dKO-specific chromatin loops is shown. The statistical significance is determined using one-sided Fisher’s exact test. (or one-sided binomial test?)

**Figure 2**. **Tcf1/Lef1-dependent changes in chromatin accessibility and super enhancer activity are concordant with changes in chromatin loop strength.**

**a.** Tcf1 peaks are more frequently associated with WT-specific ChrAcc sites than with dKO-specific ones. Differential (Diff) ChrAcc sites were identified in WT and dKO CD8+ T cells and then stratified with Tcf1 binding peaks. Values denote ChrAcc site numbers with or without Tcf1 peaks.

**b**. Tcf1 direct binding sites are preferably associated with WT- over dKO-specific ChrAcc sites. Tcf1-bound differential ChrAcc sites were stratified with the enrichment level of Tcf/Lef motif, and the sites with strong, intermediate and weak motif enrichment (Motif+, MotifInt and Motif–, respectively) were plotted as fraction of all Tcf1-bound sites specific to WT or dKO CD8+ T cells. Values denote actual numbers of ChrAcc sites in each motif enrichment group. Statistical significance between marked groups was determined with one-sided Fisher’s exact test.

**c**. ChrAcc at distal Tcf1 direct binding sites decreases upon loss of Tcf1 and Lef1. Tcf1 binding sites with different motif scores (Motif+, MotifInt, and Motif–) were identified in distal regulatory regions (>1 kb from TSS), and then analyzed separately for aggregated ChrAcc profiles in WT and dKO CD8+ T cells. The statistical significance was calculated using one-sided Wilcoxon signed rank test.

**d**. Anchors of differential chromatin loops are highly enriched with concordant differential ChrAcc sites. The enrichment scores of differential ChrAcc sites were determined at anchors of WT- or dKO-specific Chromatin (Chr) Loops. The statistical significance was calculated using one-sided Fisher’s exact test.

**e.** The changes in strength of Chr Loops are correlated with ChrAcc changes at loop anchor(s). The ratio of Chr Loop strength between WT and dKO CD8+ T cells was determined in different groups based on the presence of differential ChrAcc sites at one or both loop anchors. The blue dotted line denotes no change in loop strength.

**f.** Tcf1 peaks are enriched in super enhancers (SEs) in mature CD8+ T cells, and statistical significance is calculated using binomial test.

**g**. Tcf1 peaks are more enriched in WT-prepotent SEs compared with dKO-prepotent SEs, and statistical significance is calculated using Poisson test. In **f** and **g**, the expected numbers of Tcf1 peaks were estimated based on genome average.

**h**. The changes in strength of Chr Loops are correlated with SE activity changes at loop anchor(s). The ratio of Chr Loop strength between WT and dKO CD8+ T cells was determined in different groups based on the presence of differential SEs at one or both loop anchors. In **e** and **h**, statistical significance of indicated pairwise comparisons was determined using one-sided Mann-Whitney U test. The blue dotted line denotes no change in loop strength.

**Figure 3. Tcf1 and Lef1 are required for repressing non-T lineage enriched genes in mature CD8+ T cells**.

**a**. Functional annotation of up- and down-regulated genes in dKO CD8+ T cells with the DAVID Bioinformatics Resources. The most enriched categories and corresponding statistical significance are shown.

**b**. Heatmap showing lineage enriched genes (LEGs) for pan-T, B, pan-NK cells, conventional DC (cDC), plasmacytoid DC (pDC), monocytes (Mo) and bone marrow granulocytes (Gr) based on transcriptomic analysis of multiple immune cell lineages (GSE109125). Color scale represents z-score transformed from expression data.

**c**. pan-T cell LEGs are enriched in WT over dKO CD8+ T cells, where 61 of 153 genes are at the leading edge.

**d**. non-CD8+ T cell LEGs are enriched in dKO over WT CD8+ T cells, where 89 of 261 B cell LEGs, 83 of 239 pan-NK cell LEGs, 77 of 202 cDC LEGs, 79 of 267 pDC LEGs, 107 of 243 monocyte LEGs, and 167 of 580 granulocyte LEGs are at the leading edge. For all GSEA, enrichment plot for each gene set is shown, and also marked are NES (normalized enrichment score), NOM p-val (nominal p values), and FDR q-val (false discovery rate q values). Red and blue rectangles mark genes in the leading edge, enriched in WT and dKO CD8+ T cells, respectively. Top enriched genes in each set is shown in heatmaps.

**Figure 4. Tcf1 and Lef1 control chromatin accessibility for target gene regulation**.

**a**. Differential ChrAcc site-associated Tcf1/Lef1 target genes were displayed as Tcf1/Lef1-repressed and -activated groups in an expression heatmap (left). In each group of genes, the associated differential ChrAcc sites were clustered according to the changes in ChrAcc (middle panel). Values in parentheses denote numbers of Diff ChrAcc sites in each cluster. In the right panels, the left column displays the signal strength of Tcf1 peaks at each ChrAcc site, and the right column displays Tcf/Lef motif scores for each site as denoted with a horizontal line of distinct color code.

**b**–**f**. At select gene loci, shown are Tcf1 ChIP-seq tracks in WT and *Tcf7*–/– CD8+ T cells (top), DNase-seq tracks (middle) and H3K27ac ChIP-seq tracks (bottom) in WT and dKO CD8+ T cells. Whole or partial gene structure and transcription orientation are displayed on top of each panel. Blue horizontal bars denote MACS2-called high confidence Tcf1 binding peaks.

**b**, **c**. Tcf1/Lef1-repressed genes with increased ChrAcc sites in dKO CD8+ T cells, at promoter regions of *Gzma* and *Gzmb* (**b**) or distal regulatory regions of *Blk* and *Ctla4* (**c**), as marked by yellow vertical bars.

**d**. Tcf1/Lef1-repressed genes with decreased ChrAcc sites at distal regulatory regions of *Fasl, Cd40lg, Pax5* and *Syk* in dKO CD8+ T cells, as marked by green vertical bars. A distal site upstream of *Gzmb* in **b** belongs to this category.

**e**, **f**. Tcf1/Lef1-activated genes with decreased ChrAcc sites at promoter regions of *Gria3* and *Pou2af1* (**e**) or distal regulatory regions of *Tubb3* and *Bach2* (**f**), in dKO CD8+ T cells, as marked by blue vertical bars.

**g**. Distribution of Tcf1 peaks with different motif enrichment (Motif+, MotifInt and Motif–) in Tcf1-bound Diff ChrAcc sites in C2 and C4 clusters as defined in **a**. Values denote actual numbers of Tcf1-bound ChrAcc sites in each motif enrichment group.

**Figure 5. Tcf1 and Lef1 modulate silencer and super enhancer activity for target gene regulation**.

**a**. H3K27ac profiles at differential ChrAcc clusters C1, C2 and C4 from **Fig. 4a**.

**b**. Tcf1 ChIP-seq, DNase-seq and H3K27ac ChIP-seq tracks at the *Prdm1* locus. The 24 kb upstream Tcf1-bound ChrAcc site is in the C2 cluster, as marked with a green vertical bar. The intron 3 Tcf1-bound ChrAcc site, marked with dotted rectangle, was similar in ChrAcc signal strength in WT and dKO CD8+ T cells.

**c**. *Prdm1* expression was determined with RT-PCR in WT, *Prdm1*(Int3)M/M and *Prdm1*(–24kb)M/M naïve CD8+ T cells. Data are means+/–S.D. from ≥ 3 independent experiments (n≥5). \*\*\*, p<0.001 as determined with Student’s *t*-test.

**d**. Scatterplot showing correlation between H3K27ac changes on differential SEs and expression changes of SE-associated genes, with selected genes marked.

**e,f**. Tcf1 ChIP-seq, DNase-seq and H3K27ac ChIP-seq tracks at select SE-associated, Tcf1/Lef1-activated (**e**) or -repressed genes (**f**). Black horizontal bars under each panel denote SE-encompassed regions. Blue vertical bars denote C4 differential ChrAcc sites in WT-prepotent SEs (**e**), and yellow bars denote C1 sites in dKO-prepotent SEs (**f**).

**Figure 6. Tcf1/Lef1 TFs promote formation of chromatin interaction hubs for target gene transactivation**.

**a**. A diagram showing workflow of identifying WT- and dKO-specific chromatin interaction (Chr. Int.) hubs and hub-associated target genes.

**b**. Visualization of the output from chromatin interaction hub analysis. Shown on the left is the network containing decreased chromatin interactions connected to *Myb* promoter (up to depth 3 nearest neighbors), where the nodes represent 10-kb bins on chromosome 10 and the lines represent chromatin interactions decreased in dKO CD8+ T cells. On the right is the WT-specific hub harboring the *Myb* gene.

**c**. A cluster on chromosome 11 harboring *Ccl* gene loci formed a dKO-specific hub. In **b** and **c**, gene symbols are marked at the promoters (circle), with blue and red font denoting down- and up-regulated expression in dKO CD8+ T cells, respectively. Triangles filled with blue and red denote statistically significant decrease and increase in ChrAcc in dKO CD8+ T cells, respectively.

**d**. Tcf1 peaks are more enriched in WT-specific hubs compared with dKO-specific hubs. The expected numbers of Tcf1 peaks were estimated based on genome average, and statistical significance is calculated using the Poisson test.

**e**. Scatterplot showing correlation between the changes of interaction strength in differential chromatin interaction hubs and expression changes of their associated genes, with selected genes marked. The *x*-axis value of a dot represents the median of all interaction changes in the corresponding hub.

**f**. Two-dimensional display of changes in chromatin interaction within the *Myb*-containing hub in WT and dKO CD8+ T cells.

**g**. One-dimensional display of changes in ChrAcc and H3K27ac within the *Myb*-containing hub in WT and dKO CD8+ T cells. Blue vertical bars denote ChrAcc sites showing reduction in dKO cells.

**Figure 7. Tcf1/Lef1 TFs repress CD8+ lineage-inappropriate genes through promoting as well as disengaging chromatin interactions**.

**a–c**. Tcf1 and Lef1 repress *Ldlrad3* by promoting chromatin interactions. **a**, network display of a WT-specific hub containing the *Ldlrad3* gene. **b**, 2D display of changes in chromatin interactions within the *Ldlrad3*-containing hub in WT and dKO CD8+ T cells. **c**. 1D display of changes in ChrAcc and H3K27ac within the *Ldlrad3*-containing hub in WT and dKO CD8+ T cells. Green vertical bar denotes a ChrAcc site showing reduction in dKO cells.

**d–e**. Tcf1 and Lef1 repress *Ccl* genes by disengaging chromatin interactions, with the network display of a dKO-specific hub containing the *Ccl* genes in **Fig. 6c**. **d**, 2D display of changes in chromatin interactions within the *Ccl* gene-containing hub in WT and dKO CD8+ T cells. **e**. 1D display of changes in ChrAcc and H3K27ac within the *Ccl* gene-containing hub in WT and dKO CD8+ T cells. Yellow vertical bars denote ChrAcc sites showing increase in dKO cells.

**Figure 8. Tcf1/Lef1 TFs control multiple aspects of T cell functions**.

**a**. Validation of diminished CCR7 expression in naïve dKO CD8+ T cells with cell surface staining. Values denote geometric mean fluorescent intensity (gMFI).

**b**. T cell homing assay. CTV-labelled CD45.1+ WT and CD45.2+GFP+ dKO CD8+ T cells were mixed at 1:1 ratio (contour plot on the left) and adoptively transferred into CD45.2+ recipients via *i.v*. injection. Twenty-four hrs later, donor cells were detected in recipient LNs (contour plot on the right). Cumulative data on donor cell abundance are means ± s.d. from two experiments.

**c**. Validation of diminished EOMES expression in naïve dKO CD8+ T cells with intranuclear staining, with gMFI marked.

**d**. Detection of memory-phenotype cells in splenic GFP+CD8+ T cells from WT and dKO mice. Cumulative data on the frequency of CD44hiCD122+ cells are means ± s.d. from two experiments.

**e**. Validation of diminished MYB expression in naïve dKO CD8+ T cells with immunoblotting.

**f**. Validation of elevated FOXP3 and NRP1 expression in naïve dKO CD8+ T cells with intranuclear and surface staining, respectively, with gMFI marked.

**g**. Experimental design for testing CD8+ T cell activation and differentiation *in vivo*.

**h–j**. Detection of CD8+ T cell division and CD25 induction during the first 60 hrs of activation. CTV-labeled splenic CD8+ T cells were transferred into CD45.1+ hosts followed by LCMV infection. CTV dilution and CD25 expression were monitored before (0 hrs), 48 or 60 hrs after infection in recipient spleens. **h**, representative dot plots where vertical dotted lines mark cells in separate cell divisions. **i**, Cumulative data on the frequency of cells in indicated cell division at 48 (top) and 60 hrs (bottom) post-infection. **j**. Cumulative data on CD25 gMFI in cells at different dividing stages at 48 (top) and 60 hrs (bottom) post-infection. Data are means ± s.d. from two experiments.

**k**. Detection of effector P14 CD8+ T cells on day 8 post-infection. Effector P14 cells were detected as GFP+CD8+ T cells in recipient spleens, with values in representative contour plots denoting cell frequency. Cumulative data on effector P14 cell counts are means ± s.d. from two experiments. Statistical significance for all cumulative data was determined with Student’s *t*-test. \*, p<0.05; \*\*, p<0.01; \*\*\*, p<0.001.