



Satb1 enforces CD4⁺ effector T cell lineage stability by repressing *Foxp3* via DNA methylation

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ABSTRACT

Special AT-rich sequence binding protein 1 (Satb1) is a critical chromatin organizer that globally regulates the T lymphocyte transcriptome. While its influence on primary T cell development is well documented, defining its specific function in mature peripheral T cells has been complicated by the developmental defects inherent to early-stage deletion models. To bypass these limitations, we utilized a *Thpok-cre* conditional knockout system to delete Satb1 specifically within mature CD4 single-positive thymocytes. We demonstrate that the ablation of Satb1 results in the spontaneous emergence of an atypical CD25⁺FoxP3⁺ population from naïve peripheral CD4⁺ T cells. This aberrant de-repression of *Foxp3* occurs independently of TGFβ signaling and is fundamentally driven by alterations in DNA methylation, requiring the activity of the TET2 and TET3 demethylases. Furthermore, we show that this Satb1-deficient *Foxp3* expression is unstable during *in vitro* activation and is insufficient to confer full regulatory T cell (Treg) functionality. Collectively, our findings reveal a critical requirement for Satb1 in enforcing the lineage stability and functional fitness of conventional CD4⁺ T cells by maintaining the epigenetic silencing of *Foxp3*, providing vital insights into T cell homeostasis.

1. Introduction

Special AT-rich sequence binding protein 1 (Satb1) is a crucial transcription factor that modulates the 3D genome architecture. Because Satb1 is highly expressed in thymocytes, its function during primary T cell development in the thymus has been extensively studied. As a chromatin organizer, Satb1 regulates the global transcriptome of T lymphocytes and influences numerous aspects of T cell biology. Several genes have been identified as targets of Satb1, most notably *Foxp3* [1] (encoding FoxP3, Forkhead Box P3) and *Pdcd1* [2] (encoding PD-1, Programmed cell death protein 1), both of which have significant clinical importance in cancer immunotherapy. Since germ-line deletion of Satb1 in mice results in premature death at 3 weeks of age [3], the role of Satb1 during T cell development has been examined using conditional gene knock-out systems of *Vav-Cre* [4], *Lck-Cre* [5] or *Cd4-cre* [6,7]

transgenic strains, which eliminate LoxP-flanked target genome sequences from HSC (Hematopoietic Stem Cell), CD4⁺CD8⁺ DN (Double Negative) thymocytes or CD4⁺CD8⁺ DP (Double Positive) thymocytes, respectively. Studies using these genetic tools showed that the loss of Satb1 in T cell precursors in the thymus severely disturbed CD4⁺ helper versus CD8⁺ cytotoxic lineage choices and decreased the frequency of positively selected thymocytes [3,6], indicating that Satb1 plays an important role in functional segregation between helper and cytotoxic lineages.

Satb1 functions as a genome organizer and has been shown to generate an open chromatin structure at the Th2 cytokine locus [8] or mediate a long-distance enhancer-promoter interaction for the *Ccl5* gene [9]. Recent studies have shown that Satb1 establishes a super-enhancer region that activates the *Foxp3* locus [1] by mediating higher-order chromatin structures on multiple CNSs (conserved non-coding

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sequences) [10,11]. However, the detailed mechanism of Satb1-mediated regulation of *Foxp3* gene has yet to be fully elucidated since Satb1 appears to have differential roles in regulating *Foxp3* depending on the developmental stage [1]. The regulatory network involving FoxP3 and Satb1 is complex because FoxP3 also regulates the *Satb1* gene to elicit the suppressive activity of Treg cells [12]. One of the obstacles to deciphering the precise roles of Satb1 in immune responses is its non-negligible influence on the developmental processes of many types of primary T cells. This makes it difficult to interpret the data obtained from cells with conditional deletions of Satb1 using the Cre transgenic strains used in previous studies. For instance, the inactivation of Satb1 by *Cd4-cre*, which deletes target genes from CD4⁺CD8⁺ DP precursors, compromises the CD4⁺ helper and CD8⁺ cytotoxic lineage dichotomy, resulting in a mismatch between MHC (Major histocompatibility complex) restriction and helper/cytotoxic lineages. In this setting, the adoptive transfer of either CD4⁺ or CD8⁺ T cells into host mice alone cannot completely resolve this limitation because these T cells undergo improper development. Thus, the specific deletion of Satb1 from mature CD4 single positive (SP) thymocytes using *Thpok-cre* [13] is a better strategy for deciphering the specific roles of Satb1 in mature CD4-lineage T cells. Even with studies involving Tregs, *Thpok-cre* has its own advantage since *Thpok-cre* becomes active only from the mature CD4 SP stage, which is different from more lineage restricted *Foxp3-cre*.

Here, we report that the ablation of Satb1 in mature CD4 SP thymocytes by *Thpok-cre* resulted in the emergence of CD25⁺FoxP3⁺ cells from naïve CD4⁺ T cells. This atypical de-repression of *Foxp3* by the ablation of Satb1 was mediated in part by DNA de-methylation. Through targeted genetic approaches, we demonstrate that Satb1 is critical for maintaining the appropriate DNA methylation status necessary to silence *Foxp3* in peripheral CD4⁺ T cells, an epigenetic mechanism that relies heavily on TET2 and TET3 pathways. Furthermore, we show that this regulatory axis operates independently of TGFβ signaling. Notably, the aberrant FoxP3 expression induced by Satb1 deficiency is inherently unstable upon activation and fails to confer full regulatory T cell functionality. Together, these findings uncover a previously unappreciated role for Satb1 in actively enforcing the lineage stability and functional fitness of mature effector T lymphocytes.

2. Material and methods

2.1. Mice

Satb1^{Venus} [14], *Tet2*^{F/F}; *Tet3*^{F/F} [15], *Dnmt1*^{F/F} [16], *Foxp3*^{GFP} [17], *Satb1*^{F/F} [4], *Thpok-cre* [13] mice have been previously described. This study utilized mice of both sexes, ranging in age from 1.5 to 6 months. The animals were housed under specific pathogen-free (SPF) conditions at the RIKEN IMS facility and Kyoto University. Animal experimental protocols were conducted in strict compliance with the institutional guidelines and were formally approved by the RIKEN Yokohama Branch Institutional Animal Care and Use Committee (IACUC) under protocol 28-017, in addition to the Institutional Animal Experimental Committee of Kyoto University. Euthanasia was carried out through cervical dislocation or carbon dioxide inhalation while the mice were under anesthesia.

2.2. Cell cultures

Splenic or lymph node-derived T cells were isolated using either magnetic-activated cell sorting (Miltenyi Biotec) or fluorescence-activated cell sorting via a FACS Aria (BD Biosciences). These purified cells were then maintained in custom D-MEM (KOHJIN BIO) containing 10% fetal bovine serum (Hyclone). For standard activation, 2.0×10^5 T cells per well were seeded in 96-well plates coated with 2 µg/ml anti-CD3e (clone 553058, BD Biosciences) and supplemented with 2 µg/ml soluble anti-CD28 (clone 553295, BD Biosciences). Following a 48-h

stimulation period, the culture was supplemented with 40 U/ml rmIL-2 (R&D Systems). Retroviral transduction was achieved through spin-infection, utilizing supernatants harvested from Plat-E cells 48 h post-transfection. To induce an exhausted phenotype, T cells remained on anti-mouse CD3e/CD28 antibody-coated plates for an additional 72 h with continued IL-2 support. B16-F10 cells were cultured separately in GIBCO D-MEM with 10% FBS. All media contained 100 U/ml penicillin and 100 µg/ml streptomycin.

2.3. Quantitative RT-PCR

Total RNA was extracted using TRIzol Reagent (Thermo Fisher) and subsequently purified via Direct-zol RNA kits (Zymo Research), incorporating an on-column DNase I digestion step to ensure the removal of contaminating genomic DNA. Following purification, first-strand cDNA was synthesized using the SuperScript IV First-Strand Synthesis System (Thermo Fisher) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was then performed on a StepOne Real-Time PCR system (Thermo Fisher) using Power SYBR Green PCR Master Mix (Thermo Fisher). All primer sequences utilized in this study are available from the authors upon request.

2.4. RNA-seq and bioinformatical analysis

Transcriptomic libraries were constructed from purified mRNA using the NEBNext Ultra II RNA Library Prep Kit (NEB) in accordance with the manufacturer's instructions. Following quantification with the KAPA Library Quantification Kit (KAPA Biosystems), libraries were sequenced on an Illumina HiSeq 2000/2500 platform. The resulting sequencing data were mapped to the mouse reference genome (mm10) utilizing HISAT2 (version 2.1.0) with default parameters. Gene-level read counts were then generated using HTSeq (version 0.11.0). Statistical analysis for differential expression was performed using the EdgeR package (version 3.14) within the Bioconductor framework.

2.5. Flow cytometry and antibodies

Thymocytes and cells from peripheral lymphoid organs were isolated according to standard protocols, involving filtration through a 70 µm mesh and subsequent RBC hemolysis. The following fluorochrome-conjugated antibodies were used at a final concentration of 0.5 µg/ml: TCRβ (H57-597), CD4 (RM4-5), CD8α (53-6.7), CD8β (YTS156.7.7), CD25 (PC61), CD44 (IM7), CD62L (MEL-14), CD69 (H1.2F3), B220 (RA3-6B2), and CD24 (M1/69). Antibodies were purchased from BD Bioscience, Biolegend or Thermo Fisher. The panel also included exhaustion and activation markers: PD-1 (RMP1-30), Tim3 (B8.2C12), 2B4 (2B4), CTLA4 (L3D10), FoxP3 (FJK-16s), and IFNγ (XMG1.2). Intracellular proteins were stained following fixation and permeabilization (BD Biosciences). For intracellular staining, cell suspensions were first stained for surface antigens, washed, and then fixed and permeabilized simultaneously using BD Perm/Fix solutions (BD Biosciences) for 30 min at 4 °C in the dark. Following this initial fixation, the cells were washed twice with the kit-specific permeabilization wash buffer. Antibodies against intracellular targets were subsequently diluted directly into the permeabilization buffer and incubated with the cell suspensions for an additional 30 to 45 min at 4 °C. After the intracellular binding, the cells underwent two wash steps with the permeabilization buffer. The fully stained samples were then resuspended in standard FACS buffer prior to immediate flow cytometric acquisition. Multicolor analysis was executed on BD Symphony A3 or FACSCanto II systems, with results processed in FlowJo v10.

2.6. Statistical analysis

In all summary plots, individual data points correspond to biological replicates (independent mice), unless noted otherwise. Results are

presented as mean $\pm$ standard deviation (SD). Group comparisons were evaluated using an unpaired, two-tailed Student's t-test conducted within GraphPad Prism 10 software. Differences were defined as statistically significant when $p < 0.05$. Specific statistical parameters and sample sizes for each experiment are detailed in the respective figure legends.

3. Results

3.1. *Satb1* ablation leads to *Foxp3* derepression in peripheral $CD4^+$ T cells

To understand the function of *Satb1* in mature $CD4^+$ or $CD8^+$ lineage $\alpha\beta$ T cells, it is essential to minimize the effect of *Satb1* loss in precursor thymocytes before positive selection. Therefore, we crossed the *Satb1*^{fl^{ox}} (*Satb1*^F) mutant strain with *Thpok-cre* transgenic (Tg) strain which starts expressing Cre recombinase specifically in CD4 SP thymocytes after positive selection [13] and established *Satb1*^{F/F};*Thpok-cre* mice. *Satb1* inactivation in helper-lineage T cells by *Thpok-cre* did not alter the CD4/CD8 expression pattern as opposed to *Satb1* inactivation by *Cd4-cre*, in which $CD4^+CD8^+$ double-positive mature T cells emerged [6]. In addition, the balance of naïve/memory populations, as assessed by CD62L/CD44 expression, was not affected by the loss of *Satb1* by *Thpok-cre* (Fig. 1A), unlike *Satb1* ablation by *Cd4-cre* which resulted in an increase in the $CD44^+$ population [9]. These data suggests that the activation status of peripheral $CD4^+$ T cells was not affected when *Satb1* expression was abolished during thymocyte maturation.

Given that *Satb1* modulates genomic organization to regulate the

transcriptomes of T cells, we examined the gene expression profiles of splenic $CD4^+$ T cells by RNA-seq. At the threshold of $p < 0.01$ and $\log_2FC > 1$, only approximately 200 transcripts were differentially regulated (including both up- or down-regulated) by the loss of *Satb1* with *Thpok-cre* from peripheral $CD4^+$ T cells (Fig. 1B). No significantly enriched gene ontology (GO) terms were identified (data not shown), suggesting that *Satb1* deficiency induces selective changes in specific target genes rather than global transcriptomic changes in peripheral $CD4^+$ T cells. Consistent with previous findings [1], *Foxp3* was highly de-repressed in splenic $CD4^+$ T cells in the absence of *Satb1* (Fig. 1B), whereas other signature genes of Treg cells did not show any significant difference, including *Il2ra* (CD25), *Ikzf4* (Eos), *Cd83*, *Id3*, *Nrp1*, *Ctla4*, *Ikzf2* (Helios), *Id2*, *Foxo1* and *Foxo3*. Analyses of *FoxP3* and CD25 protein expression by flow cytometry revealed that the loss of *Satb1* resulted in a dramatic increase in the $FoxP3^+CD25^-$ population, which was accompanied by an apparent decrease and slight increase in the frequencies of the $FoxP3^+CD25^-$ and $FoxP3^+CD25^+$ populations, respectively (Fig. 1C). This change in frequencies of $FoxP3^+CD25^-$, $FoxP3^+CD25^-$ and $FoxP3^+CD25^+$ subsets suggest that de-repression of the *Foxp3* gene mainly occurs in naïve $CD4^+$ T cells that have $FoxP3^+CD25^-$ phenotype in wild-type (wt) control mice. *Foxp3* de-repression from *Satb1*^{F/F} $CD4^+$ T cells after retroviral Cre transduction (Fig. 1D) indicates that *Satb1* expression is also necessary to suppress *Foxp3* expression in *in vitro* cultured activated $CD4^+$ T cells. Taken together, the absence of *Satb1* in peripheral $CD4^+$ T cells results in the significant de-repression of *Foxp3* gene, generating an aberrant $FoxP3^+CD25^-$ population.

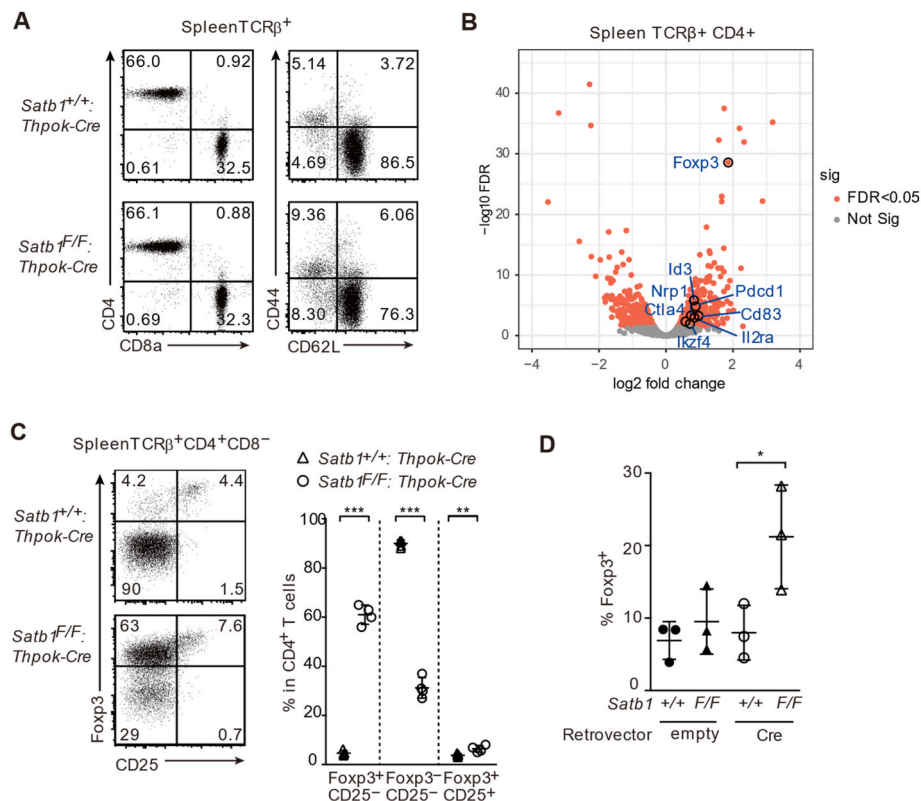


Fig. 1. Deletion of *Satb1* from $CD4^+$ T cells results in the de-repression of *FoxP3*

(A) Phenotypic characterization of splenic T cells. Flow cytometric dot plots illustrate TCRβ⁺ populations in the spleens of mice with the indicated genotypes. Data shown are representative of at least three independent experiments; percentages within each quadrant are provided. (B) Differential gene expression in splenic $CD4^+$ T cells. A volcano plot depicts the RNA-seq results from two biological replicates. Dotted lines define the significance thresholds (fold change > 2 and $p < 0.01$), with key regulatory T cell-associated genes highlighted. (C) Distribution of $CD4^+$ subsets. Representative flow cytometry profiles of splenic TCRβ⁺ $CD4^+$ $CD8^-$ T cells are shown ($n = 4$). Quadrant frequencies are indicated, and the accompanying graph on the right provides a quantitative summary. (D) In vitro *FoxP3* induction. $CD4^+$ T cells (WT or *Satb1*^{F/F}) were stimulated and transduced with either an empty vector or Cre-expressing retrovirus. *FoxP3*-GFP expression was quantified via flow cytometry ($n = 3$). P-values were determined by an unpaired two-tailed Student's t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.2. Aberrant Foxp3 expression induced by the loss of Satb1 is unstable

Our observations indicate that Satb1 expression in mature conventional CD4⁺ T cells is important for the suppression of Foxp3, although previous reports have mentioned that Satb1 expression levels were lower in peripheral Treg cells [1]. To examine how Satb1 expression levels change during Treg differentiation in the thymus, we utilized mouse strains harboring a *Foxp3*^{hCD2} [18] and *Satb1*^{Venus} reporter allele, which produces a Venus-Satb1 fusion protein [14]. By using hCD2 expression from the *Foxp3*^{hCD2} allele, the Treg developmental stage in CD4⁺ SP thymocyte population was divided into hCD2⁻CD25⁻, hCD2⁺CD25⁻ and hCD2⁺CD25⁺ (Fig. 2A). In the thymus of the male mouse harboring these two reporter alleles (*Satb1*^{Venus/+}; *Foxp3*^{hCD2}), Venus-Satb1 expression in hCD2⁻CD25⁻ cells was detected in almost a uniform manner at a high level. On the contrary, hCD2⁺CD25⁻ and hCD2⁺CD25⁺ cells showed variegated expression of Venus-Satb1. Although the peak and mean fluorescent intensity of Venus-Satb1 in the Venus-Satb1 expressing population was lower in two FoxP3⁺ subsets

than that of the FoxP3⁻ subset, a significant amount of Satb1 is still expressed in a proportion of the FoxP3⁺ population. A similar heterogeneous Venus-Satb1 expression pattern was observed in Tregs of spleen and mesenteric lymph nodes (Fig. 2B).

It should be noted that FoxP3 expression levels in activated Foxp3⁺CD25⁻ cells is not increased after 5 days of *in vitro* culture in the absence of Satb1, while FoxP3 levels in the Foxp3⁺CD25⁺ population was increased to levels comparable to control Foxp3⁺CD25⁺ cells (Fig. 2C). This observation indicates that the de-repression of Foxp3 in non-Treg cells upon the loss of Satb1 is not well-maintained during activation. Therefore, we further examined whether stable transduction of FoxP3 could induce Treg signature genes in the absence of Satb1 after activation. To test this hypothesis, we transduced the FoxP3 cDNA by retrovirus into CD4⁺FoxP3⁻CD25⁻ cells from *Satb1*^{+/+}:*Thpok-cre*:*Foxp3*^{hCD2} and *Satb1*^{F/F}:*Thpok-cre*:*Foxp3*^{hCD2} mice and examined transcripts of genes related to Treg function after 5 days of *in vitro* activation (Fig. 2D). Examination of Treg-signature genes such as *Il2r*, *Ctla4*, *Itgae*, *Gitr*, and *Helios* by the quantitative RT-PCR showed that most Treg-

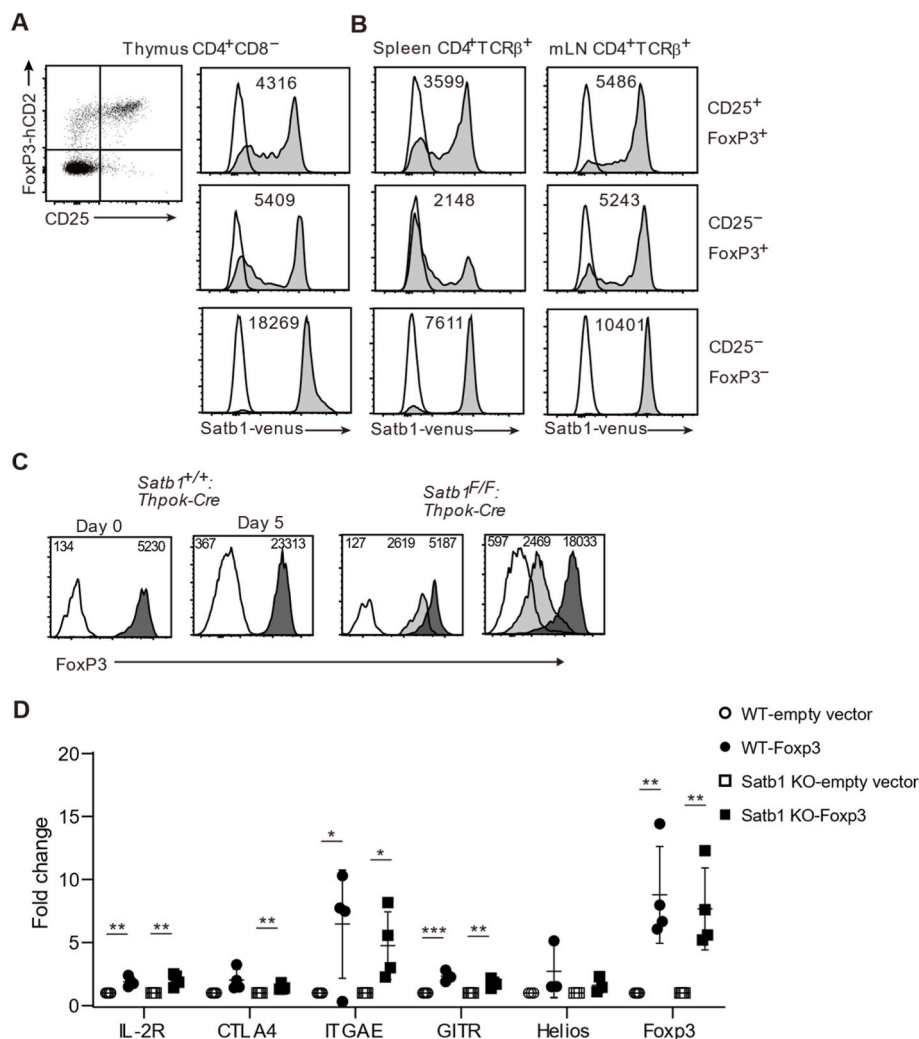


Fig. 2. FoxP3 is not stable without Satb1

(A) Monitoring Satb1 expression in thymic lineages. Satb1 protein levels were tracked using a *Satb1*-Venus reporter mouse (IRES-Venus knock-in at the 3' UTR). Thymocytes were gated by FoxP3-hCD2 and CD25 expression (left), and Venus fluorescence intensity within these subsets was assessed via histogram (right). Shaded areas represent knock-in cells, while white histograms denote WT controls. (B) Peripheral Satb1 expression. Flow cytometric profiles of splenocytes (left) and lymph node cells (right) from *Satb1*-Venus mice, analyzed as in (A). (C) FoxP3 stability in *Satb1*-deficient T cells. CD4⁺ subsets—defined as FoxP3⁺CD25⁺ (dark), FoxP3⁺CD25⁻ (grey), or FoxP3⁻CD25⁻ (white)—were isolated from WT or *Satb1*^{F/F}:*Thpok-cre* mice carrying a FoxP3-GFP reporter. Histograms illustrate GFP levels at day 0 and day 5 post-stimulation (n = 2). (D) Transcriptional effects of FoxP3 reconstitution. FoxP3-negative CD4⁺ T cells were transduced with FoxP3 cDNA retroviruses and cultured for 5 days. Expression of Treg-functional genes was quantified (n = 2 experiments in duplicate). Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001 (Student's t-test).

signature genes were successfully induced even in the absence of *Satb1*, suggesting that the expression of Treg signature genes after activation is maintained if FoxP3 is stably provided.

3.3. *Satb1* maintains the epigenetic silencing of *Foxp3* via DNA methylation

Next we examined the pathways involved in Foxp3 de-repression in naïve CD4⁺ T cells upon the loss of *Satb1*. TGFβ is known to induce FoxP3 expression through SMAD2/3 binding to CNS1 (Conserved Non-coding Sequence 1) [19], in particular for iTreg induction in culture. We therefore tested whether the loss of the TGFβ signal *in vivo* by the inactivation of the *Tgfr2* gene had any impact on the emergence of the FoxP3⁺CD25⁺ population in *Satb1*^{F/F};*Thpok*-cre mice. In *Satb1*^{F/F};*Tgfr2*^{F/F};*Thpok*-cre double mutant mice, the frequency of CD4⁺FoxP3⁺CD25⁺ in peripheral lymph nodes was comparable to that in *Satb1*^{F/F};*Thpok*-cre mice (Fig. 3A), indicating that the development of FoxP3⁺CD25⁺ cells in *Satb1*^{F/F};*Thpok*-cre is TGFβ signal-independent.

FoxP3 expression is also known to be regulated by DNA methylation status in the CNS2, also called the TSDR (Treg-specific demethylated region) [20]. DNA de-methylation and methylation are regulated by the TET family of proteins and DNMT1 maintenance methyltransferase, respectively. Among the TET family of proteins, TET2 and TET3 play major and redundant roles in T cells [21]. Therefore, we introduced Tet2^F and Tet3^F mutant alleles onto *Satb1*^{F/F};*Thpok*-cre mice by crossing them and established *Satb1*^{F/F};*Tet2*^{F/F};*Tet3*^{F/F};*Thpok*-cre triple mutant mice. The frequency of CD4⁺FoxP3⁺CD25⁺ subset became significantly lower upon the loss of TET2 and TET3 proteins (Fig. 3B). As the

reciprocal approach to TET2/3 deficiency, we examined the effect of the deletion as a reciprocal approach to DNMT1. In *Satb1*^{F/F};*Dnmt1*^{F/F};*Thpok*-cre mice, the frequency of CD4⁺FoxP3⁺CD25⁺ became significantly higher than that in *Satb1*^{F/F};*Thpok*-cre mice (Fig. 3B). These results obtained by genetic approaches indicate that *Satb1* plays an important role in the maintenance of the appropriate DNA methylation status in peripheral CD4⁺ T cells to control FoxP3 expression.

4. Discussion

CD4⁺CD25⁺FoxP3⁺ Tregs are differentiated from CD4⁺CD25⁺FoxP3⁺ population in the thymus through two alternative pathways, via CD4⁺FoxP3⁺CD25⁺ or CD4⁺FoxP3^{low}CD25⁺ precursors [22] [23]. Among these two precursors for Tregs, CD4⁺FoxP3^{low}CD25⁺ precursors give rise to Tregs with less suppressive function compared to Tregs developed from FoxP3⁺CD25⁺ precursors *in vivo* EAE model [24]. Previous studies have shown the existence of CD4⁺FoxP3^{low}CD25⁺ cells from both thymus and periphery in the absence of *Satb1*, however, the nature of these cells is not clear. Here we have characterized the aberrant CD4⁺FoxP3^{low}CD25⁺ cells emerged upon the loss of *Satb1* protein by means of *Thpok*-cre (*Satb1*^{F/F};*Thpok*-cre mice). Even though this population resembles thymic CD4⁺FoxP3^{low}CD25⁺ cells in terms of surface markers (CD4, FoxP3 and CD25), our data indicates that peripheral CD4⁺FoxP3^{low}CD25⁺ cells in *Satb1*^{F/F};*Thpok*-cre mice appear to be more or less peripheral CD4⁺FoxP3⁺CD25⁺ non-Treg cells. Therefore, our results suggest that FoxP3 expression alone in the absence of *Satb1* cannot confer the full functionality of Tregs and requires the help of *Satb1*. This is different from *in vitro* stimulated iTreg cells (Fig. 2C)

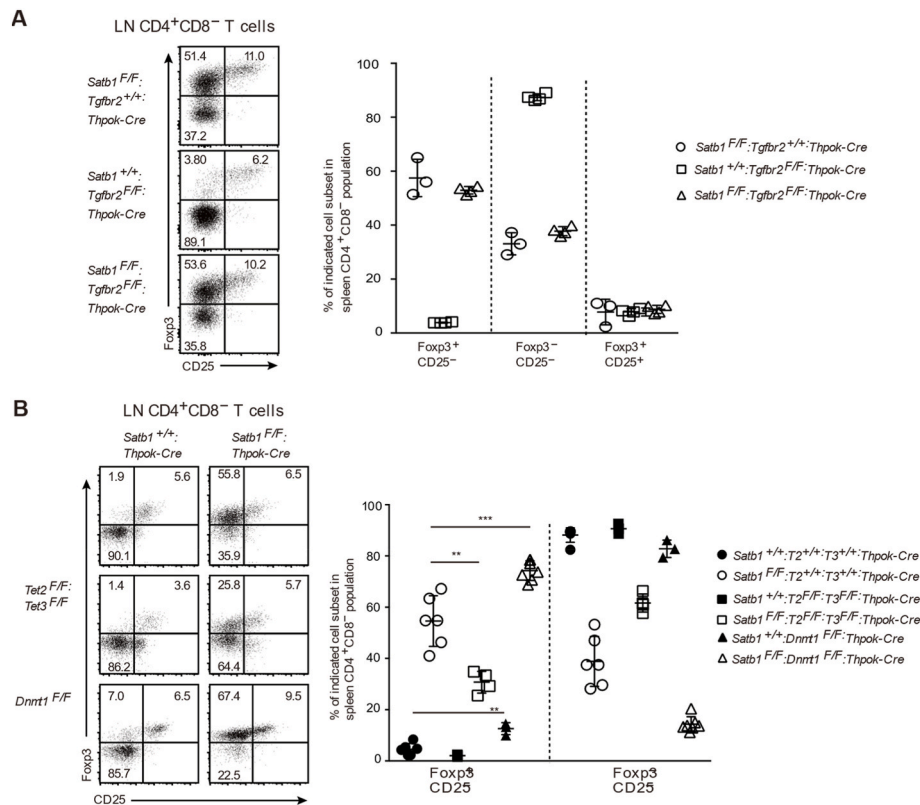


Fig. 3. *Satb1* regulates FoxP3 gene through modification of DNA methylation

(A) Genetic interaction between *Satb1* and TGFβ signaling. We assessed the combined loss of *Satb1* and *Tgfr2* (TGFβ receptor 2) by analyzing FoxP3 and CD25 expression in CD4⁺ T cells from the lymph nodes. Representative flow cytometric profiles (n = 3) are displayed with quadrant percentages. Cumulative data for FoxP3⁺CD25⁺, FoxP3⁺CD25⁻, and FoxP3⁻CD25⁺ subsets are summarized in the accompanying graphs. (B) Synergy between *Satb1* and epigenetic modifiers. The impact of simultaneous deficiency in *Satb1* and either *TET2/3* or *Dnmt1* was evaluated in lymph node TCRβ⁺CD4⁺ T cells. Bivariate dot plots show representative data from at least three animals per genotype. Statistical summaries for FoxP3⁺CD25⁺ and FoxP3⁻CD25⁺ populations are provided on the right. Significant differences are indicated: **p < 0.01, ***p < 0.001 (Student's t-test).

[25], suggesting that Satb1 provide signals which can be substituted by the downstream signaling of TCR stimulation.

The requirement for TGF β signaling for tTreg development has been controversial, but the induction of pTreg, especially iTreg, seems to clearly depend on TGF β as shown from studies utilizing *Tgfr2^{fl}/Cd4-cre* mice [26,27]. Mechanistically, the conserved region 1 (CNS1) in the *Foxp3* locus has been shown to be critical for the induction pTreg and iTreg as the platform for the binding of SMAD3, the downstream molecules of TGF β signaling [19]. TGF β also assists STAT5-mediated CNS2 demethylation during the induction of Foxp3 expression on mature Treg [28]. Satb1 binds to CNS0 and CNS3 to regulate *Foxp3* expression via chromatin modifications [1]. Therefore, it is plausible to speculate that Satb1 and TGF β bind to different CNS regions to regulate either negatively and positively *Foxp3* expression, respectively. Indeed, recent studies have indicated chromatin looping between CNS0 and CNS3 as well as between CNS0 and CNS2 [10]. However, the looping between CNS0 and CNS1 has not yet been examined. Therefore, it is unclear whether Satb1 on CNS0 interacts with SMAD3 on CNS1. In this respect, our data at least indicates that TGF β signaling pathways is not involved in *Foxp3* de-repression in the absence of Satb1. This picture is more complicated since TGF β has been shown to repress Satb1 expression from T cells [2,29] and FoxP3 also represses Satb1 [12]. Further studies will be required to decipher the detailed regulatory network among TGF β (SMAD3), Satb1 and FoxP3.

CRediT authorship contribution statement

Wooseok Seo: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Chengcheng Zou:** Investigation. **Krutula Nair:** Investigation. **Haruhiko Koseki:** Resources. **Terumi Kohwi-Shigematsu:** Resources. **Hiroyoshi Nishikawa:** Resources. **Ichiro Taniuchi:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The data for RNA-seq analysis have been deposited in GEO database under the accession code GSE190915 super series. To review GEO accession GSE190915, go to <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE190915> and enter token sropeaudvettyx into the box.

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