Identification of a novel enhancer essential for Satb1 expression in T_{H2} cells and activated ILC2s

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The genome organizer, special AT-rich binding protein-1 (SATB1), functions to globally regulate gene networks during primary T cell development and plays a pivotal role in lineage specification in CD4+ helper-, CD8+ cytotoxic-, and FOXP3+ regulatory-T cell subsets. However, it remains unclear how Satb1 gene expression is controlled, particularly in effector T cell function. Here, by using a novel reporter mouse strain expressing SATB1-Venus and genome editing, we have identified a cis-regulatory enhancer, essential for maintaining Satb1 expression specifically in T_{H2} cells. This enhancer is occupied by STAT6 and interacts with Satb1 promoters through chromatin looping in T_{H2} cells. Reduction of Satb1 expression, by the lack of this enhancer, resulted in elevated IL-5 expression in T_{H2} cells. In addition, we found that Satb1 is induced in activated group 2 innate lymphoid cells (ILC2s) through this enhancer. Collectively, these results provide novel insights into how Satb1 expression is regulated in T_{H2} cells and ILC2s during type 2 immune responses.

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Introduction

The nuclear protein, special AT-rich binding protein 1 (SATB1), functions as a genome organizer and regulates highly ordered chromatin structures by tethering specialized AT-rich genomic regions, such as base unpairing regions (1, 2). SATB1 epigenetically regulates gene expression by recruiting various chromatin modifiers and nucleosome remodelling and deacetylase (NURD) complexes (3) and promote heterochromatin formation. Multiple studies have delineated the role of SATB1 for postnatal neuronal development and function (4, 5). In addition, SATB1 is highly expressed in the thymus and is deemed essential for the development of mature thymocytes (6). CD4+CD8+ double-positive (DP) immature thymocytes undergo a TCR-mediated selection process, known as positive selection, to become mature thymocytes that are committed to become either helper-(T_{H}), cytotoxic-(T_{C}) or regulatory-T (Treg) cells. In this context, SATB1 is required to control expression of genes encoding lineage specification transcription factors, Thpok, Runx3, and Foxp3 for T_{H}, T_{C}, and Treg cells, respectively. In the absence of SATB1, aberrant expression of these transcription factors occurs through their derepression. For instance, Thpok expression was induced in MHC-I-restricted T_{C} cells and Foxp3 expression was induced in conventional CD4+ T cells (7, 8).

The functions of SATB1 extend into the differentiation of effector T cells after encountering antigens in the periphery. Satb1 expression is thought to be under the control of TCR signalling (9), which is counteracted by TGF-β signalling (10). In these effector T cells, SATB1 functions to repress PD-1 expression and suppress T cell exhaustion (10). Lastly, Satb1 expression is increased upon IL-23 stimulation in pathogenic IL-17-producing T_{H}Ts and promotes their pathogenicity in experimental autoimmune encephalomyelitis via regulation of GM-CSF production and suppression of PD-1 (11). The role of SATB1 in CD4+ T_{H}2 differentiation has been characterized but has found very contradictory results. Based on the identification of SATB1-binding sites in the T_{H}2 locus containing Il-5, Il-4, and Il-13 genes in a murine T_{H}2 cell line in vitro, the first report claimed that SATB1 functioned as a positive regulator of T_{H}2 cytokine expression (12). Other studies, however, demonstrated that SATB1 only represses IL-5 expression in human CD4+ T_{H}2 cultures (13). It was also reported that SATB1 cooperates with β-catenin to control the expression of Gata3, the key T_{H}2 lineage transcription factor critical for T_{H}2 differentiation and function (14). However, conditional loss of Satb1 in Cd4+ T cells, using the ThPOK-Cre mouse strain, showed that loss of Satb1 expression had no detrimental effects on T_{H}2 differentiation, at least in vitro. Furthermore, it has been shown that the expression of Satb1 is controlled by both IL-4 and NFκB signalling in vitro (15).

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Figure 1. Expression of SATB1-venus reporter during T cell development and activation.

(A) Schematic shows Satb1-Venus gene locus in Satb1Venus knockin mice.

(B) Histograms, on the left, show expression of SATB1-Venus in CD4–CD8– DN, CD4+CD8+ DP, CD4–CD8– TCRβhiCD4+CD8– mature SP and CD4–CD8– TCRβhiCD4–CD8+ mature SP thymocytes of Satb1+/Venus mice. Numerical values indicate SATB1-VENUS MFI (mean fluorescence intensity) and data are a representative of three biological experiments and summarised in the middle graph. Statistics were calculated by two-way ANOVA Tukey’s multiple comparisons: *p < 0.05, **p < 0.01, ***p < 0.001.

Graph, on the right, shows Satb1 mRNA expression in DN3, DP, CD4 SP and CD8 SP thymocytes, derived from IMMGEN RNA-seq database.

(C) Histograms show SATB1-Venus expression in conventional CD4+CD25- versus regulatory CD4+CD25+ mature thymocytes of Satb1+/Venus mice. Data are representative of three biological experiments.

(D) Histograms, on the left, show expression of SATB1-Venus in splenic B cells (B220+TCRβ–), T cells (B220– TCRβ+), CD4 T cells (TCRβ+CD4+CD8α–), and CD8 T cells (TCRβ+CD4–CD8α+) of Satb1+/Venus mice. Data are summarised in the middle graph from three biological experiments and statistics were calculated by two-way ANOVA Tukey’s multiple comparisons: *p < 0.05, **p < 0.01, ***p < 0.001.

Graph on the right shows Satb1 expression in B cells, naive CD4, and naive CD8 T cells from IMMGEN RNA-seq database. (E) Histograms show SATB1-Venus expression in CD4

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Currently, there is very little mechanistic insight to how Satb1 gene expression is transcriptionally induced in T cells. In mice, there are four alternative promoters, P1, P2, P3, and P4 generate Satb1-1α, Satb1-1b, Satb1-1c, and Satb1-1d transcripts, respectively. Usage of these four Satb1 promoters is differentially regulated in CD4 T cells in vitro (15, 16). Interestingly, a GWAS study mapped a Psoriasis-associated single nucleotide polymorphism (SNP, rs73178598) around 240 kb upstream of the Satb1 locus, where an antisense noncoding RNA called SATB1-AS1 is transcribed (17). This study hinted the presence of cis-regulatory genomic elements that could control Satb1 expression in various effector T cells and contribute to tissue inflammation. Thus, it is quite important to define Satb1 expression during effector T cell differentiation and how it is controlled.

In this study, we used a novel Satb1-Venus reporter strain and a genome editing technology to identify a novel IL-4-responsive Tν2-specific enhancer for Satb1, defined as Satb1-Eth2. Satb1-Eth2 is essential to maintain Satb1 expression, not only in CD4 T cells but also in activated ILC2s. Furthermore, loss of Satb1-Eth2 resulted in elevated IL-5 expression in CD4 T cells. Collectively, our study unravels mechanisms by which Satb1 expression is retained in immune cells mediating type 2 immune responses.

Results

Strict control of Satb1 expression revealed by a Satb1-Venus fusion reporter

To quantify SATB1 protein expression in various types of murine cells, particularly in T cells and at single cell level, we generated a Satb1-Venus allele by the knock-in approach to insert the Venus open reading frame, downstream of the initiation codon (in exon2) of the Satb1 gene (Figs 1A and S1A and B). In Satb11/2/Venus thymocytes, the Satb1-Venus allele expressed the SATB1-Venus protein (Fig S1C) that showed a cage-like distribution in the nucleus, as previously reported (Fig S1D) (18). Phenotypic analyses in Satb1+/Venus mice did not show abnormalities in thymocyte development, that was observed in Satb1+/Venus mice, and the numbers of mature CD4 and CD8 SP thymocytes were comparable with the wildtype (Fig S1E). Thus, the SATB1-Venus fusion protein is likely to retain endogenous SATB1 function to support, at least, primary T cell development. Using Satb11/2/Venus mice, we observed that most of the CD4+CD8− DN thymocytes expressed low levels of SATB1-Venus (Figs 1B and S1F). Further analyses of DN sub-populations revealed that SATB1-Venus is initially lowly expressed in CD4+CD25−DN1 thymocytes, and it is then incrementally increased with DN1 transition into CD44+CD25−DN2 and CD44+CD25+DN3 thymocyte stages (Fig S1F). In CD25+CD44− DN thymocytes, there were SATB1-Venus+ and SATB1-Venus− populations, the latter of which is likely to be non-T lymphoid cells. There was also a significant increase in the number of SATB1-Venus+ DP thymocytes, during their transition from CD4−CD8− DN to CD4+CD8− DP stage (Fig 1B). Notably, DP thymocytes undergoing positive selection (CD69+TCRβhigh and CD69+TCRβlow) showed further increase in SATB1-Venus expression (Fig S1G), which may suggest that SATB1 expression, is positively controlled by TCR stimulation. In addition, there was a significant decline in SATB1-Venus expression from positively selected DPs to mature thymocytes (Figs 1B and S1G). Moreover, analyses of Satb1-Venus transcript levels in DN3, DP, CD4 SP, and CD8 SP thymocytes from the immunological genome project (ImmGen, https://www.immgen.org/) (19) were highly consistent with the SATB1-Venus protein levels (Fig 1B). These data suggest that our Satb1-Venus reporter mouse model can be used to endogenously study the SATB1 protein function. In thymic Tregs, defined as CD24+TCRβhighCD4+CD8−CD25+ cells, SATB1-Venus expression was lower than their conventional CD4 counterparts (Fig 1C). Further analyses of nonconventional T cell subsets, such as the invariant natural killer T (iNKTs) and γδ T cells, showed that iNKTs cells expressed similar levels of SATB1-Venus to that in conventional CD4 SP cells but was significantly higher than in γδ T cells (Fig S1H).

We next analysed SATB1-Venus expression in peripheral lymphocytes in the spleen. Both CD4+ and CD8+ T cells in the spleen showed higher SATB1-Venus expression than B cells (Fig 1D), which again showed high consistency with the Satb1 transcript levels (https://www.immgen.org/) (19). When CD4+ T cells were further gated for naive (CD62LhighCD44high), central memory (CD62LhighCD44low), and effector memory (CD62LlowCD44high) populations, we noted a uniform and high SATB1-Venus expression in naive CD4 T cells (Figs 1E and S1I). On the other hand, there were SATB1-Venus+ and SATB1-Venus− populations in both CD4 central memory and CD4 effector memory populations, with increased frequency of SATB1-Venus− in effector memory cells. In the CD8 T cell pool, both naive and central memory populations sustained a uniform and high SATB1-Venus expression, whereas the effector memory T cells consisted of both SATB1-Venus+ and SATB1-Venus0 populations. These observations suggest that T cell memory differentiation in the periphery induces down-regulation of SATB1 expression.

Intestinal intraepithelial lymphocytes (IEL) are most likely to represent effector T cells, as they are continuously exposed to various antigens in the gut. In contrast to splenic T cells of Satb1−/Venus mice, 85% of which are SATB1-Venus+ cells, only 2.5% of the intestinal TCRβ− IEL were SATB1-Venus+ (Fig 1F). This SATB1-Venus+ IEL population expressed CD62L and showed similar CD4 and CD8α expression profiles to that in SATB1-Venus+ splenic T cells. These data suggest that these CD62L+SATB1-Venus+ TCRβ+ IEL are recent immigrants of circulating αβ T cells. On the contrary, the or CD8 T cell subpopulations: CD62LhiCD44hi naive, CD62LhiCD44hi central memory, and CD62LloCD44hi effector memory T cells from Satb1−/Venus mice. Numerical values indicate SATB1-Venus MFI and data are a representative of four biological experiments. Data are summarised in the adjacent graph. (F) Contour plots show SATB1-Venus and CD62L expression in splenic versus intraepithelial TCRβ+ T cells of Satb1−/Venus mice, and the CD4 and CD8α expression patterns on CD62L+ SATB1-Venus+ and CD62L−SATB1-Venus− T cell populations. Data are representative of three biological experiments. (G) Histograms show SATB1-Venus expression in naive CD4 T cells undergoing activation in vitro for 0, 24, 48, and 72 h and summarised in the adjacent graph from three biological experiments. Statistics were calculated by unpaired t test; *P < 0.05, **P < 0.01, ***P < 0.001. (H) Histograms show SATB1-Venus expression in naive CD4 T cells versus in vitro differentiated CD4 Tαβ, T−αβ, T−β, and T−γδ T cells from Satb1−/Venus mice. Data are representative of three biological experiments and summarised in the adjacent graph. Statistics were calculated by two-way ANOVA Tukey’s multiple comparisons: *P < 0.05, **P < 0.01, ***P < 0.001.
CD62L*SATB1-Venus* TCRβ* IEL mainly represented a mixture of CD4+, CD4*CD8α* and CD4*CD8α* subpopulations and most of the CD4*CD8α* cells were nonconventional CD8α* IELs (data not shown). This, therefore, suggests that SATB1-Venus could serve as a good marker for separating effector and naive T cells.

To examine SATB1 expression during T cell activation, SATB1-Venus was quantified in *Satb1*/*Venus* naive CD4 T cells undergoing TCR activation in vitro. Indeed, SATB1-Venus levels significantly elevated after 24- and 48-h post activation and plateaued after 72 h post activation (Fig 1G). Furthermore, we traced SATB1-Venus expression in various effector CD4 helper T cell subsets that were differentiated in culture. Interestingly, SATB1-Venus expression is highest in CD4 Tg1, Tg2, and Tg17 and lowest in Tregs (Fig 1H). Overall, SATB1 expression in T cells is dynamically regulated during thymocyte development and in their functional differentiation into effector cells, in a cell-context-dependent manner.

**Identification of cis-regulatory genomic regions for *Satb1* gene regulation**

Our *Satb1-Venus* reporter mice revealed that the amount of SATB1 protein is significantly altered during T-cell development and transcriptome data generated by the ImmGen project (https://www.immmgen.org/) (19) shows a nice correlation of SATB1-Venus in T cell subsets with activated/memory characteristics (Fig 2A, and publicly available ATAC-seq data (19) to search for open chromatin shows higher chromatin accessibility (21). Therefore, we used CD4+CD8α- mutant mice in vitro, under Th0, Th1, Th2, Th17 or iTreg polarising conditions. Interestingly, we observed a substantial reduction in SATB1-Venus expression levels were significantly reduced in naive CD4 T cells of Satb1+/Venus-Δb mutant mice (Fig 2B). It was also noteworthy that loss of Satb1-a caused a significant reduction in SATB1-Venus expression in CD4 effector-memory populations but had no effects on CD8 naive and memory T cell populations. These results suggest that Satb1-a is essential for maintaining Satb1 expression in peripheral CD4 T cells with activated/memory phenotypes.

In addition to the decline of Satb1-a and Satb1-b have any roles in regulating SATB1 expression in effector CD4 T cell subsets, we also detected naive CD4 T cells of Satb1+/Venus-Δa and Satb1+/Venus-Δb mutant mice in vitro, under Th0, Th1, Th2, Th17 or iTreg polarising conditions. Interestingly, we observed a substantial reduction in SATB1-Venus expression in Satb1+/Venus-Δa CD4 Tg0 and Tg17, but not strikingly, SATB1-Venus expression was almost lost in Satb1+/Venus-Δb Tg2 cells (Fig 2C). Therefore, the Satb1-a enhancer may allow to maintain high levels of SATB1 expression in Tg2 cells. On the contrary, we did not detect any decline in SATB1-Venus in Satb1+/Venus-Δb CD4 Tg0, Tg1, Tg2, Tg17 or iTreg cells (Fig 2C), thereby discarding any potential roles of Satb1-b in regulating Satb1 expression in T cells. We also confirmed that removal of the Satb1-a region from the Satb1 locus resulted in a significant reduction of SATB1 protein levels in vitro-differentiated CD4 Tg2 and Tg0 cells (Fig S2D), which was accompanied with reduction of Satb1 transcripts (Fig 2D). These results confirm that Satb1-a is essential to maintain Satb1 expression under Tg2 polarising conditions.

**Satb1-a region functions as a genomic enhancer**

In addition to the decline of Satb1 expression, we also observed a significant decline in the expression of Gm19585 transcripts in Satb1+/Venus-Δa CD4 Tg2 cells (Fig 2D). Therefore, one would speculate whether the loss of Gm19585 transcripts, which may function as noncoding RNAs, were causative for impaired Satb1 expression. Hence, to mechanistically understand how Satb1-a controls cis expression of Satb1 in CD4 Tg2 cells, we examined whether the noncoding Gm19585 RNAs are primarily involved in stabilising Satb1 expression. To this aim, we performed Gm19585 knockdown studies in Tg2-polarised CD4 T cells in vitro. Analyses of Gm19585 transcripts deposited on UCSC genome browser showed that there are at least eight alternatively spliced transcripts, as some transcripts were either exon1- or exon2-depleted sequences. Therefore, three shRNA
Figure 2. Analyses of Satb1-a and Satb1-b function in regulating Satb1 expression.

(A) ATAC-seq and H3K27Ac ChIP-seq signals in Satb1, and in upstream genomic regions transcribing Gm20098 and Gm19585 in various murine T cell populations indicated. Red dashed boxes highlight genomic regions, Satb1-a and Satb1-b, that show ATAC-seq and/or H3K27Ac ChIP-seq signals in thymocytes and/or T cells. Satb1-a and Satb1-b are evolutionarily conserved and located near regions transcribing Gm19585 and Gm20098, respectively. 

(B) Histograms show SATB1-Venus expression in CD4 T cells.

(C) SATB1-Venus expression in CD8 T cells.

(D) Satb1 and Gm19585 expression in various T cell populations.
expression plasmids, pLKO-Gfp-Gmj585-1, pLKO-Gfp-Gmj585-2, and pLKO-Gfp-Gmj585-4, that target exon1, exon2, and exon4 of Gmj585 transcript respectively, were generated (Fig S2E). In vitro-activated CD4 T cells were transduced with these shRNAs expressing lentiviral particles and were maintained in T42 polarising conditions for additional 6 d. shRNA-transduced CD4 T42 cells defined as CD4+GFP+ were sorted and were subjected to Satb1 transcriptional analyses by qRT-PCR. pLKO-Gfp-Gmj585-2 or pLKO-Gfp-Gmj585-4 shRNAs were able to knockdown Gmj585 transcripts by twofold but had no significant effects on Satb1 transcript levels in CD4 T42 cells (Fig 3A). These results suggest that the noncoding RNAs from Gmj585 are unlikely to be responsible for maintaining Satb1 expression under T42 polarising conditions.

The above finding raised the possibility that Satb1-a functions as an enhancer to control Satb1 gene expression in cis. To investigate this possibility, we used a publicly available three-enzyme Hi-C (3e Hi-C) dataset from murine embryonic stem cells, thymocytes, and CD4 T42 cells (22, 23), to identify genome wide interactions between Satb1-exon1 (which contain promoters) and Satb1-a region. Interestingly, we found that Satb1-exon1 and Satb1-a are closely located within a topologically associated domain, in both thymocytes and CD4 T42 cells (Fig 3B). This indicates that a specific cis-genomic interaction between these regions is induced specifically in T cells, through chromatin looping. For further validation, we performed a chromatin capture conformation (3C) assay to confirm whether this chromatin interaction between Satb1-exon1 and Satb1-a specifically occurs in CD4 T42 cells. For this study, we designed an anchor primer AS2 for capturing Satb1-a and one primer S1 to capture Satb1 exon1b, an alternative Satb1 promoter that has been reported to be highly expressed in CD4 T42 cells (15) (Fig 3C); the primers AS1 and S2 were used as negative controls. As expected, the use of AS2-S1 primers strongly produced significant amplicon signals in CD4 T42 cells than in CD4 T0 and T1 cells. Therefore, our 3C assay validated that the chromatin interaction between Satb1-a and Satb1-a genomic regions is formed in CD4 T42 cells, to regulate Satb1 gene expression.

Given that the regulation of chromatin looping is often mediated by a transcription factor, we next sought to identify which T42-specific transcription factor could be responsible for promoting chromatin looping of Satb1-a to the Satb1 promoters in CD4 T42 cells. It is already established that the signal transducer and activator of transcription 6 (STAT6) is activated downstream of IL-4 signalling in CD4 T42 cells and directly regulates Satb1 expression (15). We noticed that there are STAT DNA-binding motifs within the Satb1-a region. Hence, we examined publicly available STAT6 ChIP-seq datasets in murine CD4 T42 cells (24) and found that Satb1-a was indeed occupied by STAT6 in these cells, suggesting that STAT6 binds and activates the Satb1-a in T42 cells (Fig 3D). Therefore, we have classified Satb1-a as an enhancer of Satb1 expression in the context of T42 and henceforth shall be referred as Satb1-Eth2 (enhancer for T42 cells).

Satb1-Eth2 is essential in repressing IL-5 expression in CD4 T42 cells in vitro

Having established that Satb1-Eth2 is essential in maintaining Satb1 expression in CD4 T42 cells, we then investigated the relevance of Satb1-Eth2 on the differentiation and function of CD4 T42 cells. For this aim, we established Satb1Eth2/–ΔEth2 mice on C57BL6 background by genome editing. First, it was previously reported that Satb1 functions to promote Gata3 expression in CD4 T42 cells in vitro (14). Therefore, we first considered whether Satb1-Eth2 had any role in regulating Gata3 expression in CD4 T42 cells in vitro. Interestingly, although Satb1Eth2/–ΔEth2 CD4+ T42 cells showed a significant loss of Satb1 mRNA, there was no significant reduction in Gata3 expression (Fig S3A). These data suggest that low levels of Satb1, via loss of Satb1-Eth2, did not impair Gata3 expression and primary CD4 T42 differentiation in vitro. Next, it has been reported that Satb1 binds and regulates the expression of the T42 cytokines IL-4, IL-5, and IL-13 (12), and hence, we examined whether Satb1-Eth2 is essential for the expression of IL-4, IL-5, and IL-13 in Satb1Eth2/–ΔEth2 CD4 T42 cells in vitro. PMA and ionomycin stimulation in Satb1Eth2/–ΔEth2 CD4 T42 cells revealed no significant differences in the intracellular expression of IL-4, which suggested that Satb1-Eth2 is not required for IL-4 induction in CD4 T42 cells (Fig S3B). We, however, did find a significant increase in IL-4 “IL-5” cells in our Satb1Eth2/–ΔEth2 CD4 T42 cultures (Fig 3A). The increase in IL-4 “IL-5” cells in our Satb1Eth2/–ΔEth2 CD4 T42 cultures was also recapitulated upon TCR stimulation (Fig S3C). Further analyses of IL-4, IL-5, and IL-13 transcripts in PMA and ionomycin-stimulated Satb1Eth2/–ΔEth2 CD4 T42 cells revealed that only IL-5 transcripts were significantly elevated in Satb1Eth2/–ΔEth2 CD4 T42 cells (Fig S3D). Moreover, the elevated expression of IL-5 in Satb1Eth2/–ΔEth2 CD4 T42 cells coincided with elevated IL-5 secretion in culture supernatants post PMA and ionomycin stimulation (Fig 3B). These results collectively show that Satb1-Eth2 is crucial to maintain SATB1 levels in CD4 T42 cells, and to restrain IL-5 expression levels.

Satb1-Eth2 is required to maintain SATB1 expression in CD4 T42 and ILC2s during T42 immune responses in vivo

We have demonstrated that Satb1Eth2/–ΔEth2 CD4 T42 cells have elevated the expression of IL-5 in vitro. These results are consistent with former studies showing the requirement of SATB1 in suppressing IL-5 in human CD4 T42 cells (13). IL-5 is the key soluble factor for eosinophil activation and recruitment. At the site of a T42 splenic CD4 or CD8 T cell subpopulations (1. CD62L+CD44hi naive, 2. CD62L-CD44hi central memory, and 3. CD62L-CD44hi effector memory T cells) from Satb1-Venus, Satb1–/–ΔEth2, and Satb1Venus–/– mutant mice. Numerical values indicate SATB1-Venus MFI (mean fluorescence intensity). Graph shows quantification of SATB1-Venus MFI in splenic CD4 and CD8 T cell subpopulations. Data are summarised from four biological experiments. Statistics were calculated by two-way ANOVA Tukey’s multiple comparisons: *P < 0.05, **P < 0.01, ***P < 0.001. (C) Histograms show SATB1-Venus expression in vivo differentiated CD4 T0, T1, T2, T17, and iTReg cells, generated from Satb1Eth2/–ΔEth2 (top) and Satb1Eth2/–ΔEth2 (bottom) mutant mice. Data are a representative of at least two biological experiments. Adjacent graphs show quantification of SATB1-Venus MFI. Data are summarised from two or three biological experiments. (D) qRT–PCR analysis of Satb1 and Gmj585 expression in Satb1–/–ΔEth2 and Satb1Venus–/– CD4 T0, T1, T2, cells. Data are summarised from four biological experiments. Statistics were calculated by unpaired t test; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
immune response, eosinophils release granules containing proinflammatory proteins that cause inflammation and tissue damage. Dysregulation in IL-5 expression and eosinophil activity drive allergic reactions and have clinical implications in the progression of asthmatic responses. We therefore speculated that SATB1 functions to repress CD4 TH2 immune responses via IL-5 suppression and promotes immune resolution. Thus, we next explored the in vivo function of Satb1-Eth2 in controlling type 2 immune responses and used an extract of *Alternaria alternata* (*A. A.*) to induce experimental airway inflammation. First, we traced eosinophils in infiltration in the bronchial alveolar lavage (BAL) after treating the Satb1+/Venus mice with *A. A.* on days 4, 7, and 10, and confirmed it to peak from day 7 to 10 (Fig S4A). On day 7, we found a small but significant induction of SATB1-Venus in CD4 T\(_{h2}\) cells (Fig S4B), which is further maintained at day 10. During this study, we also noticed that SATB1-Venus expression was induced in ILC2s (Fig S4B), a subset of lymphocytes responsible for T\(_{h2}\) responses at the early phase of lung inflammation. Previous studies failed to detect Satb1 expression in ILC2s of naive mice, so we suspected whether ILC2s could only induce Satb1 expression during lung inflammation. Indeed, ex vivo analyses of lung resident ILC2s in naive mice revealed very little expression of SATB1-Venus expression (Fig S4B).

![Image](https://example.com/image1)

**Figure 3.** Satb1-a interacts with Satb1 promoter and is bound by STAT6. **(A)** Graphs show qRT–PCR analyses of Gm19585 and Satb1 expression in CD4 T\(_{h2}\) cells transduced with pLKO-GFP-empty, pLKO-Gfp-Gm19585-1, pLKO-Gfp-Gm19585-2 or pLKO-Gfp-Gm19585-4 shRNA-expressing vectors. Data are representative of at least two biological experiments. **(B)** Heatmaps show contact matrices of chromosome 17 from murine embryonic stem cells (top) versus thymocytes (middle) and CD4 T\(_{h2}\) cells (bottom). The location of Satb1-exon1 and Satb1-a are pinpointed by black arrows. **(C)** Schematic shows the locations of Satb1-exon1 and Satb1-a loci and primer design for the 3C assay. The positions of primers S1 and S2 overlap Satb1-exon1b and Satb1-exon1a, respectively. The position of anchor primers AS1 and AS2, respectively, align outside and nearby the Satb1-a region. The primer pairs S1-AS1, S1-AS2, S2-AS1, and S2-AS2 were used in combination to capture interactions between Satb1-exon1 and Satb1-a. Data are summarised in the graph below, from two biological experiments. **(D)** Genome browser ChIP-seq tracks show binding of STAT6 to the Satb1-a in murine CD4 T\(_{h2}\) cells. ATAC-seq signals in colonic Tregs are shown as reference. Two putative STAT6-binding motifs within the Satb1-a region are indicated as grey ovals.
mice failed to up-regulate SATB1-Venus expression (Fig 5A). Crucially, these results demonstrate the strict requirement of Satb1-Eth2 in inducing SATB1 expression during in vivo T(H)2 immune responses. To determine whether loss of Satb1-Eth2 function exacerbated lung inflammation in response to A.A., we examined eosinophil infiltration in BAL of A.A.-treated Satb1ΔEth2/ΔEth2 mice versus Satb1+/+ but found no significant increase in eosinophil numbers in A.A.-treated Satb1 ΔEth2/ΔEth2 mice at 7 d post-injection (Fig 5B). In addition, quantification of IL-5 in the BAL supernatants of A.A.-treated Satb1 ΔEth2/ΔEth2 mice also revealed no significant increase in IL-5 expression (Fig 5B). Alternatively, we also used the OVA induced lung inflammation model to induce T(H)2 immune responses in Satb1 ΔEth2/ΔEth2 mice (Fig S4D) but again found no significant increase in eosinophil numbers and in IL-5 concentrations in the BAL of OVA-challenged Satb1 ΔEth2/ΔEth2 mice (Fig S4E). Lastly, sorted lung CD4 T(H)2 cells and ILC2s from A.A.-treated mice, at 10 d post-injection, showed similar frequencies of IL-4+IL-5+ or IL4−IL-5+ subpopulations between Satb1+/+ and Satb1 ΔEth2/ΔEth2 mice (Fig 5C). Hence, our in vivo models could not find a functional relevance of Satb1-Eth2 in restraining both T(H)2 immune responses and IL-5 expression in CD4 T(H)2 cells and ILC2s during A.A.-induced lung inflammation. Overall, our results from the A.A.-induced lung inflammation model confirmed that Satb1-Eth2 is required to maintain SATB1 expression in innate and adaptive T(H)2 lymphocytes during acute lung inflammation, but the physiological relevance of Satb1-Eth2 in regulating T(H)2 immune responses remains to be further investigated.

Discussion

A recent GWAS study identified a Psoriasis-associated single nucleotide polymorphism in SATB1-AS1, an antisense RNA that is encoded 240 kb upstream of the SATB1 locus and showed a T-cell-specific interaction with the SATB1 promoter (17). This study was not only the first to reveal the possibility of cis-regulatory transcriptional mechanisms that could regulate Satb1 expression in both human and murine T cells, but it was also the first-link SATB1 to allergy and inflammatory disease. Previous studies have examined the function of SATB1 in T(H)2 cells. SATB1 was shown to bind to the murine T(H)2 locus and promoted Il-5, Il-4, and Il-13 gene expression (12). Another study showed the induction of Satb1 expression upon IL-4 signalling in
in vitro-differentiated CD4 T_{H2} cells (15), in a STAT6-dependent manner. This up-regulation of Satb1 expression in CD4 T_{H2} cells is associated with altered Satb1 promoter usage from P1 to P2 and P3, but the biological relevance of these promoters in regulating Satb1 expression during T cell development was not fully addressed. Hence, it was unclear how Satb1 expression is specifically controlled during effector CD4 T_{H2} cell differentiation, particularly in CD4 T_{H2} cells. In addition, current studies have not examined whether Satb1 was induced during activation of ILC2s, in part because of low Satb1 expression in steady state ILC2s. Here, we have identified and shown a crucial function of Satb1-Eth2 in regulating Satb1 expression in TH2 cells and ILC2s in vitro and in vivo. Loss of Satb1-Eth2 function significantly impacted on the transcriptional levels of Satb1, specifically in T_{H2} cells and activated

Figure 5. Satb1-Eth2 functions to regulate SATB1 expression in CD4 T_{H2} cells and activated ILC2s in vivo.

(A) Histograms show SATB1-Venus expression in BAL CD4+ T cells (CD45+TCRβ+CD4+), in lung CD4 T_{H2} cells (CD45+TCRβ+CD4+GATA3+ST2+), and in lung ILC2s (CD45+TCRβ+CD4+GATA3+ST2+) of naive and AA-treated Satb1^{+/+}, Satb1^{+/Venus} and Satb1^{+/Venus-Eth2} mice on day 7. Data from AA-injected mice are a representative of three biological replicates. Graphs summarise SATB1-Venus expression in those cells from AA-treated Satb1^{+/+}, Satb1^{+/Venus} and Satb1^{+/Venus-Eth2} mice on day 7. (B) Dot plots show frequencies of eosinophils in BAL of naive (Satb1^{+/+}) and AA-treated Satb1^{+/+} and Satb1^{+Venus-Eth2} mice on day 7. Graphs summarise eosinophil numbers and IL-5 concentration in BAL of naive (Satb1^{+/+}) and AA-treated Satb1^{+/+} and Satb1^{+Venus-Eth2} mice on day 7. Data are summarised from at least three biological replicates. (C) Contour plots show the intracellular expression of IL-4 and IL-5 in PMA and ionomycin-stimulated lung CD4 T_{H2} cells and ILC2s, from AA-treated Satb1^{+/+} and Satb1^{+Eth2/Eth2} mice on day 10. Graphs show the frequencies of IL-4^+IL-5^-, IL-4^+IL-5^+, and IL-4^-IL-5^- subpopulations in PMA- and ionomycin-stimulated lung CD4 T_{H2} cells and ILC2s from AA-treated Satb1^{+/+} and Satb1^{+Eth2/Eth2} mice on day 10. Data are summarised from three biological replicates. Statistics were calculated by unpaired t test; *P<0.05, **P<0.01, ***P<0.001.
ILCs. Interestingly, Satb1-Eth2 overlapped with exon 4 of the noncoding gene, Gm19585, and loss of Satb1-Eth2 function was accompanied with the reduction of Gm19585 transcript levels. However, knockdown of Gm19585 transcripts did not affect Satb1 expression in T\(_\text{i,2}\) cells, which suggests that the Gm19585 transcripts have no role in regulating Satb1 gene expression. Rather, chromatin looping between Satb1-Eth2 with Satb1 promoters and STAT6 binding to the Satb1-Eth2 in T\(_\text{i,2}\) cells support the notion that Satb1-Eth2 functioned as an IL-4 inducible T\(_\text{i,2}\)-specific enhancer for Satb1 gene expression.

Loss of Satb1-Eth2 expression significantly caused depression of IL-5 in T\(_\text{i,2}\) cells in vitro, which therefore highlights a biological role of Satb1-Eth2 in suppressing IL-5 expression in T\(_\text{i,2}\) cells. Our study is also consistent with what others have observed with Satb1 knockdown assays in human CD4 T\(_\text{i,2}\) cells (13), and therefore indicates that IL-5 de-repression, caused by loss of the Satb1-Eth2 function, is mediated by the reduction of SATB1. Notably, IL-5 is known to play a major role in promoting eosinophil recruitment and promote the progression of allergy or asthma (25). A previous report presumed that SATB1 had a significant role in controlling TH2 immune responses in vivo. However, these results do not formally exclude the involvement of SATB1 in controlling TH2 immune responses in vivo. Indeed, we examined the role of Satb1-Eth2 in augmenting lung inflammation in vivo but found that loss of the Satb1-Eth2 had no additive effects on eosinophil infiltration or on IL-5 expression in the BAL of AA-treated mice, albeit of low Satb1 expression in both lung T\(_\text{i,2}\) cells and ILCs. Based on this key information, one would have to truly consider whether SATB1 has any function in controlling T\(_\text{i,2}\) immune responses in vivo. However, these results do not formally exclude the involvement of SATB1 in controlling T\(_\text{i,2}\) immune responses in vivo under different experimental settings, for instance in chronic lung inflammation and in atopic-dermatitis models (26), and thus would merit further investigation.

We also uncovered a partial but significant reduction of SATB1-Venus expression in naive and effector memory CD4 T cells, caused by loss of Satb1-Eth2 function. Naive CD4 T cells undergo homostatic proliferation to maintain their survival in the periphery, which crucially requires both self-MHC ligands and the IL-7 cytokine (27). In addition, effector memory T cells require IL-15 for their survival. Both IL-7 and IL-15 signalling pathways activate the STAT5 transcription factors downstream. It should be noted that most of the STAT transcription factor members recognise the palindromic DNA sequence TTCNNNGAA. It is therefore possible that, in both naive and effector memory CD4 T cells, STAT5 could bind to Satb1-Eth2 and regulate Satb1 expression. However, because loss of Satb1-Eth2 function had a partial effect on SATB1 expression in naive CD4 T cells, we believe that Satb1-Eth2 is partially responsive to either IL-7 and/or IL-15. Alternatively, the regulation of Satb1 expression in naive and memory CD4 T cells could be mediated by other cis- or trans- enhancers, which may require an in-depth analysis.

Overall, the present study identifies and demonstrates the essential role of the cis-regulatory enhancer, Satb1-Eth2, in regulating Satb1 expression, specifically in CD4 T\(_\text{i,2}\) cells and ILC2s, which significantly contributes to our current understandings of how Satb1 expression is controlled during T\(_\text{i,2}\)-mediated immune responses. Our study signifies the beginnings of an era of identifying cis- and/or trans-regulatory elements that regulate Satb1 gene expression in T cells and thus provides insightful information on Satb1 function in a T cell context-dependent manner.

### Materials and Methods

#### Mice

The Satb1\(^{\text{Venus}}\) allele was generated by knock-in insertion of the Venus open reading frame downstream of transcription start site (exon2) of the Satb1 gene. For this aim, a BAC clone B6Ng01-312L13, which included the 5′ area of the Satb1 gene, was purchased from RIKEN BRC. A 13-kb genomic region harboring the ATG start codon was subcloned into the pBlueScript II vector (Stratagene). The DNA fragment harboring partial exon2 sequences and Venus cDNA sequences were generated by overlapping the PCR technique and was used to construct a targeting vector. The targeting vector was transfected into murine M1 ES cells as previously described (28). G418-resistant ES clones were screened for homologous recombination event between the target vector and the Satb1 gene. Appropriate ES clones were then used to generate chimera mice by ES aggregation. F1 founders from the chimera mice carrying the Satb1\(^{\text{Venus}}\) allele were selected for establishing mouse line and were analysed. Satb1\(^{\text{Fix}}\) CD4-cre mice were previously described in (7). Satb1\(^{+/\text{Venus}}\) or Satb1\(^{−/−}\text{Venus}\) and Satb1\(^{+/\text{Venus}}\) or Satb1\(^{−/−}\text{Venus}\) mice were generated by co-injection of Cas9-mRNA with sgRNAs into Satb1\(^{+/\text{Venus}}\) fertilised eggs that were generated by in vitro fertilisation with sperm from a Satb1\(^{+/\text{Venus}}\) male mouse and Satb1\(^{−/−}\) females. The F0 founders that had deleted Satb1-a or Satb1-b regions were then crossed with C57/B6N mice and F1 founders that deleted Satb1-a or Satb1-b on the Satb1\(^{\text{Venus}}\) allele were selected as heterozygous mice with the Satb1\(^{+/\text{Venus}}\) or Satb1\(^{+/+}\) or Satb1\(^{−/−}\) genotype. Among the two and three F1 Satb1\(^{+/\text{Venus}}\) or Satb1\(^{+/−}\) or Satb1\(^{−/+}\) founders, we chose one line as a representative for Satb1\(^{+/\text{Venus}}\) or Satb1\(^{+/−}\) or Satb1\(^{−/+}\) genotype and Satb1\(^{−/−}\) or Satb1\(^{+/−}\) or Satb1\(^{−/+}\) and were examined with littermate controls. Similarly, C57/B6N Satb1\(^{+/a}\) (Satb1\(^{\text{Eth2}}\)) mice were generated by co-injection of Cas9-mRNA with Satb1-a gRNA into C57Bl/6 fertilised eggs. Sequences for sgRNA are as follows: Satb1\(^{+/a}\) sgRNAs 5′-TCAACTAGAATTTGCTTC-3′ and 5′-CAGCTACACATAGATCTTC-3′; and Satb1\(^{+/b}\) sgRNAs 5′-ACACACTGCTGTGCTG-3′ and 5′-GCTGTCCTTTAGACTC-3′. All mice were maintained at the RIKEN Center for Integrative Medical Sciences. The animal protocol was approved by the institutional Animal Care and Use Committee of RIKEN Yokohama Branch (2020-028). For all experimental procedures, at least two to three mice were used per experiment and are shown either as a representative or as a summary of these biological experiments.

#### Immunohistochemistry

50,000 of thymocytes from Satb1\(^{+/+}\) or Satb1\(^{+/+}\) mice were resuspended in 0.1 ml RPMI-1640 medium (10% FBS) and were mounted on a poly-L-lysine-coated glass slide (REF: 26414; Polysciences, Inc.) and incubated at 37°C for 2 h. The attached cells were gently washed three times in PBS. The cells were fixed with 4% PFA at room temperature for 10 min and then washed three times in PBS. The cells were
permeabilized with 70% ethanol and kept at −20°C until analysis. On the
day of the analysis, thymocytes were stained with 1/100 of DAPI
(ab228549; Abcam) at room temperature, for 30 min, and washed
three times in PBS. Cover slips were mounted on top of the thy-
mocytes with Fluoromount-G mounting medium (00-4958-02;
Thermo Fisher Scientific) and sealed with Biotium CoverGrip Cov-
erslip Sealant (23005; REF). Confocal microscopic images were
obtained with TCS-SP5 (Leica Microsystems) using 40x objective.

Ex vivo cell preparation for flow cytometry

Thymus, spleen, and peripheral lymph nodes (axillary, inguinal, and
cervical) were removed from mice at 4–8 wk of age and were
mashed through a 70-μm cell strainer in a Petri dish to make single-
cell suspensions. For the elimination of red blood cells, splenocytes
were treated with ACK lysis buffer (Gibco A1049201, 2.5 ml per
spleen) for 3 min and pelleted by centrifugation at 300g at 4°C for
5 min. Supernatants were discarded and cell pellets were resus-
pended into RPMI-1640 (2% FBS). All cell suspensions were
maintained at 4°C for 5 min with continuous shaking. To homogenize the samples, the
cell suspensions from spleen, pLNs, IEL, and lungs were treated with rat
mouse CD16/CD32 (BD Bioscience) for 10 min at 4°C. Fluo-
rophore antibodies for extracellular markers were then added and
stained for 30 min at 4°C. After incubation, cells were then pelleted
by centrifugation at 300g at 4°C for 5 min and resuspended in FACS
buffer containing 7AAD/DAPI cell viability dye. For analyses of both
extracellular and intracellular markers, cell suspensions of all
tissues were stained with fixable live/dead cells (65-0866-18;
Ebiosciences) for 10 min before rat anti mouse CD16/CD32 treatment
and extracellular marker staining. Using the FOPX3 staining buffer
kit (00-5523-00; Ebiosciences), stained cells were then fixed, per-
meabilised, and stained for intracellular transcription factors as per
the manufacturer’s instructions. All cytometry analyses were per-
formed using a FACS CANTO II (BD-Bioscience) and data were
analysed using FlowJo (Tree Star) software.

In vitro CD4 T helper differentiation

Naïve CD4 T cells were pooled from spleen and peripheral lymph
nodes. The spleen and peripheral lymph nodes (axillary, inguinal,
and cervical) were removed from mice at 6–10 wk of age and were
mashed through a 70-μm cell strainer in a Petri dish to make single-
cell suspensions. Splenocytes were the treated with ACK lysis buffer
(Gibco A1049201, 2.5 ml per spleen) for 3 min before pelleting (by
centrifugation at 300g at 4°C for 5 min) and resuspension into
RPMI-1640 (2% FBS). Naïve CD4 T cells from either sorted by FACS
Aria (sorted for CD4+CD25+CD69+CD44+) or isolated by using
EasySep mouse naïve CD4 T cell isolation kit (ST-19765). Naïve T cells
were resuspended in complete medium (KOHJIN BIO DMEM-H
[16003016] + 10% FBS) and activated in the presence of 2 μg/ml
of anti-CD3 (REF: 533058; BD Pharmingen) and 2 μg/ml of anti-CD28
(REF: 553295; BD Pharmingen) (precoated in a rounded 96-well
plate) in T0,0 (5 μg/ml of anti-IFNγ [REF: 16-741185; Invitrogen], 5 μg/
ml of anti-IL-12/23 [REF: 505308; BioLegend], 5 μg/ml of anti-IL-4
[REF: 554385; BD Pharmingen] and 10 ng/ml of IL-2 [402-ML-010;
R&D systems]), T1,1 (5 μg/ml of anti-IL-12/23 [REF: 505308; BioLegend], 5 μg/ml of anti-IL-4
[REF: 554385; BD Pharmingen] and 10 ng/ml of IL-2 [402-
ML-010; R&D systems]), T2,2 (5 μg/ml of anti-IL-12/23, 10 ng/ml of IL-2, and 10 ng/ml of IL-4 [402-
ML-020; R&D systems]), T3,3 (R and D systems, CDK017, as per
the manual’s instructions), and iTReg (5 μg/ml of anti-IFNγ, 5 μg/ml of
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anti-IL-12/23, 2, 2, 3 μg/ml of anti-IL-4, 2, 2, 3 μg/ml of anti-IL-12/23, 2, 2, 3 μg/ml of anti-IL-4, 2, 2, 3 μg/ml of

and 0.5 μg/ml of ionomycin (10634–1MG; Sigma-Aldrich), in a rounded 96-well plate for 5 h in the presence of 2 μM of monesin (Cay1648-1; Biozol). T₁7 cells were stimulated as per the manufacturer’s instructions (CDK017; R and D systems). After stimulation, cells were analysed for intracellular expression of IFN-γ, IL-4, and IL-17 by flow cytometry. The in vitro differentiated Tregs were analysed for the intracellular expression of FOXP3 by flow cytometry. After confirmation of CD4 helper differentiation, Satb1 expression levels were analysed by flow cytometry, immunoblotting, and qRT-PCR. Culture supernatants of PMA and ionomycin-treated CD4 T₀ and CD4 T₁2 cells (in the absence of monesin) were also recovered, flash-frozen in N₂, and stored in −80°C. The quantification of miL-4 and miL-5 cytokines in these supernatants was performed by the Laboratory for Immunogenomics (RIKEN, IMS) via Luminex analysis.

**Western blotting**

T cells in complete medium were pelleted down by centrifugation at 200g for 5 min at 4°C. Supernatants were aspirated, and the cell pellets were resuspended in 1 ml of ice-cold PBS. Cells were transferred into 1.5 ml Eppendorfs and pelleted down by centrifugation at 200 g for 5 min at 4°C. The supernatants were aspirated and pellets were washed in 1 ml of ice-cold PBS. The cells were pelleted down by centrifugation at 200 g for 5 min at 4°C and supernatants were aspirated. Cells were lysed in lysis buffer (2% SDS in 50 μM Tris–HCL pH 8, supplemented with EDTA-free protease inhibitor, Roche) at cell concentration of 20 × 10⁶/ml and incubated at 95°C for 15 min. Debris were pelleted down by centrifugation at 13,000 g for 10 min at room temperature and protein lysates were transferred into new 1.5 ml Eppendorf tubes. Protein lysates were adjusted with 2X laemil sample buffer (Bio-Rad) + 2-mercaptoethanol and boiled at 95°C for 5 min. Supernatants were aspirated, and the cell pellets were washed in 1 ml of ice-cold PBS. The samples were vortexed for 20 s and incubated at room temperature for 3 min. The samples were pelleted down by centrifugation at 12,000 g at 4°C for15 min. The top aqueous phase containing RNA was transferred into a new tube. RNA samples were purified by using Zymogen RNA clean and concentrator kit (R1017; 2μm research), as per the manufacturer’s instructions. RNA concentrations were obtained on a NanoDrop Spectrophotometer and 1.2 μg of RNA were subjected to cDNA synthesis by using the Super VILO cDNA synthesis kit (RE1.1756050; Thermo Fisher Scientific). qRT–PCR was performed using Power up SYBR green master mix (REF: A25742; applied biosystems) on the QuantStudio three Real-Time PCR Systems, accordingly to the manufacturer’s instructions. The following gene expression levels analysed by qRT–PCR are listed in Table 1.

**Gm19585 shRNA lentiviral production**

The RNAi consortium was used to generate three shRNAs for the knockdown of Gm19585 derived transcripts (see Table 2). shRNA sequences were cloned into the PLKO.gfp cloning vector, which was kindly gifted by Dr. Jun Huh (Harvard University): cloning, transfection, and production of lentiviral particles were performed accordingly to Addgene’s protocol. After screening for shRNA inserts, lentiviruses for individual shRNAs were produced in HEK293T cells plated in six-well plates in antibiotic-free DMEM (Gibco, supplemented with glutamine and 10% FBS). For one transfection assay and using the Fugene HD transfection reagent (REF: E1312; Promega) accordingly to the manufacturer’s instructions, the HEK293T cells were cultured in 10% FBS-containing DMEM and were transfected with 1 μg of shRNA plasmid, 750 ng of pSfPAX2 packaging plasmid, and 250 ng of pMD2.G envelope plasmids. Packaging and envelope plasmids were obtained from Addgene. Viral supernatants were collected

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**Table 1. List of cDNA primers used for qRT-PCR.**

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<thead>
<tr>
<th>cDNA primer name (mouse)</th>
<th>Sequence</th>
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<td>Satb1-F</td>
<td>CCCCCTAGAAAGGAAGGCC</td>
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<tr>
<td>Satb1-R</td>
<td>GTTCACCCACAGGATGAACTGG</td>
</tr>
<tr>
<td>Gm19585-F</td>
<td>CCCGTCTAAAGGATGGAATTGGA</td>
</tr>
<tr>
<td>Gm19585-R</td>
<td>GGCATCACACTAAAGATACCCA</td>
</tr>
<tr>
<td>Hprt-F</td>
<td>GTCTGATTTAGGATGTAACCC</td>
</tr>
<tr>
<td>Hprt-R</td>
<td>ATGACATCTTCGAAGATCTTTCAG</td>
</tr>
<tr>
<td>Gata3-F</td>
<td>CGAAGACCCGAGCTTACAA</td>
</tr>
<tr>
<td>Gata3-R</td>
<td>GTCTGACAGTGCCGAGGAA</td>
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<tr>
<td>Il4-F</td>
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<td>Il5-R</td>
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<td>Il13-F</td>
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<td>Il13-R</td>
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3 d post transfection and filtered through 0.45 μm low-protein-binding filters. Polybrene (Sigma-Aldrich) was added to the viral supernatants at a final concentration of 2 μg/ml, aliquoted, and stored at ~80°C.

**Gm19585 knock-down in in vitro Tn2 CD4 T cells**

CD4 T cells from wild-type mice were isolated from pLNs (axillary, inguinal, and cervical) by using EasySep mouse CD4 T cell isolation kit (ST-19752). Cells were stimulated with 2 μg/ml of immobilised anti-CD3 and anti-CD28 in TH2 polarising conditions (5 μg/ml of anti-IL-12/23, 10 ng/ml of IL-2, and 10 ng/ml of IL-4) for 24 h. After activation, cells were pelleted down by centrifugation at 200 g, at room temperature for 5 min and resuspended in 20 μl of prewarmed PBS. The cells were added to 500 μl of thawed viral supernatants and put into 48-well plates. The cells were cultured incubator for 1 h 1 volume of 2X TH2 cytokines (5 μg/ml of anti-IL-12/23, 10 ng/ml of IL-2, and 10 ng/ml of IL-4) were then added and the cells were incubated for another 48 h. After activation, cells were pelleted down by centrifugation at 200g, at room temperature for 5 min and resuspended in 20 μl of prewarmed PBS. The cells were added to 500 μl of thawed viral supernatants and put into 48-well plates. The cells were centrifuged at 660 g at 32°C for 1.5 h and were rested in the tissue culture incubator for 1 h 1 volume of 2X Tn2 cytokines (5 μg/ml of anti-IL-12/23, 10 ng/ml of IL-2, and 10 ng/ml of IL-4) were then added and the cells were incubated for another 48 h. pLKO-Gfp (empty) and pLKO-Gfp-Gm19585 shRNA-transduced cells were monitored and TH2 cytokines were refreshed every second. CD4+GFP+ cells were then sorted on 6 d post transduction and in 20 trifugation at 200 g.

**ATAC-seq analyses**

ATAC-Bigwig files from Immgen database (19) were analysed and visualized on the IGV genome browser. Genomic regions of interest were then further analysed on the USCS genome browser for conservation.

**3e HiC data analyses**

3e HiC datasets from mESCs (GSM1620000) and CD4 Tn2 cells (GSM1619998) were downloaded from the Gene Expression Omnibus (GEO) site with the accession numbers GSE66343 (22). HiC datasets from thymocytes (GSM2868043) were downloaded from GEO site with the accession number GSE173476 (23). To examine the chromosome structure and topologically associated domain structures surrounding Satb1 and Satb1-a (exon 4 of Gm19585) loci, reads on chromosome 17 were extracted and realigned on mouse genome mm10 using bowtie2 (version 2.4.6). Mapped reads were collected and converted into.hic format using Juicer tools (version 1.22.01, https://github.com/aidenlab/juicer) and visualized using Juicebox (version 1.6, https://github.com/aidenlab/juicebox/).

**H3K27 acetylation and STAT6 ChIP-seq analyses**

Publicly available H3K27Ac ChIP-seq datasets from murine CD4+ FOXP3+ and CD4+FOXP3- T cells (8) were used to analyse H3K27Ac peaks at the Satb1-a and Satb1-b loci. The Sequence Read Archive (SRA) files SR5385345 and SRR5385345 were downloaded from SRA website. Similarly, STAT6 ChIP-seq in CD4 Tn2 cells (24), was used to analyse STAT6 DNA-binding sites at the Satb1-a locus and the SRA file SRA054075 was downloaded from SRA website. Fastdump was used to convert all SRA files into fasta, which were processed for the removal of adaptor sequences and then realigned to mm10 reference genome using Bowtie2. Samtools was then used to sort and convert the SAM files (output files, generated from the genome alignment) into BAM files. BAM files were then indexed using samtools for the generation of bigwig files. The bigwig files were then visualized on the IGV genome browser.

**3C assay**

Procedures for 3C are described in our previously published article (29). The primer sequences used for the 3C assay qRT-PCR are provided in Table 3.

**A. alternata induced airway inflammation model**

8–12 wk old female mice were anesthetized by isoflurane inhalation, followed by intra-nasal or intra-tracheal injection of A.A. extract (ITEA, 10117, 20 μg per head, in 40 μl of PBS) on Day 0, Day 3, Day 6, and/or Day 9. After 24 h post final challenge, naïve and A.A.

### Table 2. List of shRNA primers used for Gm19585 knockdown study.

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<th>shRNA primer name (mouse)</th>
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<td>Gm19585-ex1 shRNA-R:</td>
<td>AATTCATTTAAATCGACTTTTATTTCTTCTGAGTAATAAGTAAATGACTCTGCTTTTG</td>
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<tr>
<td>Gm19585-ex2 shRNA-F:</td>
<td>CCGGCACTTCTATCCCGTTACGAGTATTTACGAGTAATAAGTAAATGACTCTGCTTTTTG</td>
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<td>Gm19585-ex2 shRNA-R:</td>
<td>AATTCATTTAAATCGACTTTTATTTCTTCTGAGTAATAAGTAAATGACTCTGCTTTTG</td>
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<tr>
<td>Gm19585-ex4 shRNA-F:</td>
<td>CCGGACACGATCTATTTAATTTCTTCTGAGTAATAAGTAAATGACTCTGCTTTTTG</td>
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<td>Gm19585-ex4 shRNA-R:</td>
<td>AATTCATTTAAATCGACTTTTATTTCTTCTGAGTAATAAGTAAATGACTCTGCTTTTG</td>
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### Table 3. List of primers used for 3C qRT-PCR.

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<td>S2:</td>
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<tr>
<td>AS1:</td>
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<tr>
<td>AS2:</td>
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<td>Gapdh F:</td>
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<tr>
<td>Gapdh R:</td>
<td>GCTTGCCCTACCCCTTCCTT</td>
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treated female mice were euthanized by CO₂ inhalation. For cytokine analyses, BAL fluid (BALF) was first collected by intratracheal insertion of a catheter and 1 lavage of 500 μl of HBSS (2% FBS) and transferred into new 1.5 ml Eppendorf tubes. The extracted BALF was immediately pelleted down by centrifugation (300g, 5 min at 4°C) and BALF supernatant were transferred into new 1.5 ml Eppendorf tubes. The pelleted cells were resuspended in 500 μl of HBSS (2% FBS). BALF supernatants were flash-frozen in liquid N₂ and stored in −80°C. Additional two lavages of 500 μl of HBSS (2% FBS) were used to further collect the BALF of naïve and AA challenged mice and were combined with BALF cells from the first lavage, totalling to a final volume of 1.5 ml. BALF cells were kept on 4°C for flow cytometric analyses. Lung cell suspensions from mice were prepared as outlined above. After Percoll gradient centrifugation, 5,000 ILC2s (CD45+Lin−Thy1.2+CD4+ST2+) and ILC2s (CD45+Thy1.2+CD4−ST2+) were sorted using FACS Aria. 10,000 CD4+Thy1.2+ cells and ILC2s were activated with PMA (100 ng/ml) and ionomycin (0.5 μg/ml) in a rounded 96-well plate for 4 h in the presence of Monesin and were then subjected to flow cytometry analyses for intracellular cytokines levels. The quantification of the mIL-5 cytokine in BALF supernatants of naïve and AA-treated mice were also performed by the Laboratory for Immunogenomics (RIKEN, IMS) via Luminex analysis.

**OVA-induced airway inflammation model**

8–12-wk-old male and female mice were first immunized with intraperitoneal administration of 50 μg of OVA (in 100 μl PBS) + 100 μl of Alum (Inject Alum Adjuvant, Thermo Fisher Scientific), on Day 0 and Day 13. On days 27, 28, and 29, the mice were anesthetized by isoflurane inhalation, followed by intranasal injection of 50 μg of OVA (in 40 μl PBS). After 24 h post final challenge, BAL and lungs of naïve and OVA-challenged mice were processed and analysed using the same methods for AA-induced airway inflammation model.

**In vitro activation of ILC2s**

Lung cell suspensions from mice were prepared as outlined above. After Percoll gradient centrifugation, 5,000 ILC2s (CD45+Lin−CD3e−Thy1.2+) were sorted into each well of 96-well-plate. Sorted ILC2s were cultured in 100 μl of complete medium (KOHIJM BiDMEM-H [16003016] + 10% FBS) in the presence of mIL-33 (10 ng/ml), mIL-2 (10 ng/ml) and mIL-7 (10 ng/ml) for 4 d.

**Statistical analysis**

Student T tests and two-way ANOVA with post hoc Bonferroni tests were performed using GraphPad Prism software: ***p < 0.001, **p < 0.01, and *p < 0.05.

**Supplementary Information**

Supplementary Information is available at https://doi.org/10.26508/Isa.202301897.

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**Author Contributions**

A Nomura: formal analysis, investigation, and writing—original draft, review, and editing.

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M Ohno-Oishi: formal analysis.

K Kakugawa: formal analysis.

S Muroi: methodology.

H Yoshida: resources, formal analysis, and methodology.

TA Endo: resources, software, and formal analysis.

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I Taniuchi: conceptualization, supervision, and writing—original draft, review, and editing.

**Conflict of Interest Statement**

The authors declare that they have no conflict of interest.

**References**


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