An anti-silencer- and SATB1-dependent chromatin hub regulates Rag1 and Rag2 gene expression during thymocyte development.

https://escholarship.org/uc/item/5jf6852w

The Journal of experimental medicine, 212(5)

0022-1007

Hao, Bingtao
Naik, Abani Kanta
Watanabe, Akiko
et al.

2015-05-01

10.1084/jem.20142207

Peer reviewed
An anti-silencer– and SATB1-dependent chromatin hub regulates Rag1 and Rag2 gene expression during thymocyte development

Bingtao Hao,1 Abani Kanta Naik,1 Akiko Watanabe,1 Hirokazu Tanaka,2 Liang Chen,1 Hunter W. Richards,3 Motonari Kondo,1 Ichiro Taniuchi,2 Yoshinori Kohwi,3 Terumi Kohwi-Shigematsu,3 and Michael S. Krangel1

1Department of Immunology, Duke University Medical Center, Durham, NC 27710
2RIKEN Centre for Integrative Medical Sciences, Yokohama, Kanagawa 230-0045, Japan
3Life Sciences Division, Lawrence Berkeley National Laboratory, University of California, Berkeley, Berkeley, CA 94720

Abbreviations used: 3C, chromosome conformation capture; ASE, anti-silencer element; BAC, bacterial artificial chromosome; ChIP, chromatin immunoprecipitation; ChIP-seq, ChIP-sequencing; DP, double positive; FAIRE, formaldehyde-assisted isolation of regulatory elements; H3K4me1, histone H3 lysine 4 monomethylation; H3K4me3, histone H3 lysine 4 trimethylation; H3K27ac, histone H3 lysine 27 acetylation; H3K27me3, histone H3 lysine 27 trimethylation; RAG, recombination activating gene; RNA Pol II, RNA polymerase II; RSS, recombination signal sequence; SATB1, special AT-rich binding protein 1; SP, single positive; V(D)J, variable, diversity, and joining gene segment.

Rag1 and Rag2 gene expression in CD4+CD8+ double-positive (DP) thymocytes depends on the activity of a distant anti-silencer element (ASE) that counteracts the activity of an intergenic silencer. However, the mechanistic basis for ASE activity is unknown. Here, we show that the ASE physically interacts with the distant Rag1 and Rag2 gene promoters in DP thymocytes, bringing the two promoters together to form an active chromatin hub. Moreover, we show that the ASE functions as a classical enhancer that can potently activate these promoters in the absence of the silencer or other locus elements. In thymocytes lacking the chromatin organizer SATB1, we identified a partial defect in Tcra gene rearrangement that was associated with reduced expression of Rag1 and Rag2 at the DP stage. SATB1 binds to the ASE and Rag promoters, facilitating inclusion of Rag2 in the chromatin hub and the loading of RNA polymerase II to both the Rag1 and Rag2 promoters. Our results provide a novel framework for understanding ASE function and demonstrate a novel role for SATB1 as a regulator of Rag locus organization and gene expression in DP thymocytes.

The diverse antigen receptor repertoires of T and B lymphocytes are generated by a site-specific DNA recombination process that assembles antigen receptor variable (V), diversity (D), and joining (J) gene segments in developing lymphocytes. This process, known as V(D)J recombination, is initiated by a protein complex composed of recombination activating genes 1 and 2 (RAG1 and RAG2), which can recognize and cleave at recombination signal sequences (RSSs) that flank TCR and immunoglobulin V, D, and J gene segments (Schatz and Swanson, 2011). The Rag1 and Rag2 genes display a distinctive, tightly linked genomic organization with stringently and coordinately regulated expression during T and B lymphocyte development (Kuo and Schlissel, 2009).

There are two developmental windows of Rag1 and Rag2 (hereafter, Rag) gene expression during T and B lymphocyte development (Kuo and Schlissel, 2009). In developing thymocytes, the Rag genes are first expressed at the CD4−CD8− double-negative (DN) stage to promote recombination of the Tcrb, Treg, and Tcrd genes. Productive Tcrb recombination causes Rag gene down-regulation, cellular proliferation, and differentiation to the CD4+CD8+ double-positive (DP) stage. Rag genes are then reexpressed in DP thymocytes to promote recombination of Tcra genes. After productive Tcra gene assembly and positive selection of TCR–expressing DP thymocytes, Rag genes are silenced during differentiation to the CD4−CD8+ or CD4+CD8− single-positive (SP) stage. In similar fashion, an initial phase of Rag gene expression

© 2015 Hao et al. This article is distributed under the terms of an Attribution-Noncommercial-Share Alike-No Mirror Sites license for the first six months after the publication date (see http://www.rupress.org/terms). After six months it is available under a Creative Commons License (Attribution-Noncommercial-Share Alike 3.0 Unported license, as described at http://creativecommons.org/licenses/by-nc-sa/3.0/).
in pre-pro- and pro-B cells mediates recombination of \(IgH\) genes, whereas a subsequent phase of \(Rag\) gene expression in small pre-B cells mediates recombination of \(IgK\) and \(IgL\) genes.

Transcriptional regulation of the \(Rag\) genes is complex, involving distinct sets of lineage- and stage-specific cis-elements that cooperate with the \(Rag1\) and \(Rag2\) promoters (Kuo and Schlissel, 2009). \(Rag\) expression in developing B cells depends on sequences upstream of the \(Rag2\) promoter, including a proximal enhancer at −2.6 kb, a distal enhancer at −8 kb, and the well-studied Erag enhancer at −23 kb (Hsu et al., 2003; Kuo and Schlissel, 2009). Sequences within 10 kb of \(Rag2\) appear capable of supporting \(Rag\) gene expression in DN thymocytes (Yannoutsos et al., 2004). Elimination of this so-called anti-silencer element (ASE) by gene targeting reduced \(Rag1\) and \(Rag2\) expression by two orders of magnitude in DP thymocytes and prevented differentiation into SP cells, but had only modest effects on \(Rag\) gene expression in DN thymocytes (Yannoutsos et al., 2004). Studies with transgenic mice also revealed the presence of a silencer element between the \(Rag1\) and \(Rag2\) genes, which can extinguish \(Rag\) expression in DP thymocytes and partially suppress expression in DN thymocytes. Importantly, ASE activity was shown to be essential to counteract the suppressive effects of the intergenic silencer in DP thymocytes (Yannoutsos et al., 2004). Silencer activity depends on a binding site for \(Runx\) transcription factors, but further mechanistic information about ASE or silencer function has been lacking.

Gene regulation by distal elements generally depends on long-range interactions that are facilitated by chromatin architectural proteins (Gibcus and Dekker, 2013; Merkenschlager and Odom, 2013). Special AT-rich binding protein 1 (SATB1) is a nuclear matrix/scaffold-associated DNA-binding protein that participates in the maintenance of chromatin architecture and regulates the expression of a large number of genes that participate in the maintenance of chromatin architecture (Gibcus and Dekker, 2013; Merkenschlager and Odom, 2013). Special AT-rich binding protein 1 (SATB1) is a nuclear matrix/scaffold-associated DNA-binding protein (Skowronska-Krawczyk et al., 2014). SATB1 can also recruit chromatin remodeling complexes that either promote or inhibit gene expression (Kumar et al., 2006). Given these activities, it is not surprising that SATB1 regulates gene expression programs in a wide variety of cells, including embryonal stem cells (Savarese et al., 2009), neuronal cells (Balamotis et al., 2012), epithelial progenitor cells (Fessing et al., 2011), pituitary cells (Skowronska-Krawczyk et al., 2014), and many tumors (Kohwi-Shigematsu et al., 2013). Within the hematopoietic compartment, SATB1 is expressed in stem cells and is then up-regulated during their commitment to lymphoid lineages (Satoh et al., 2013; Will et al., 2013). In hematopoietic stem cells, SATB1 maintains quiescence and the potential for long-term self-renewal (Will et al., 2013). In hematopoietic progenitor cells, SATB1 supports the expression of genes critical for lymphocyte development and plays an important role in lymphopoiesis (Satoh et al., 2013). SATB1 is expressed at unusually high levels in the thymus (Dickinson et al., 1992), and \(Satb1\)-null mice display inefficient T cell development with the major block at the DP stage, resulting in dramatically reduced numbers of SP thymocytes and peripheral T cells (Alvarez et al., 2000; Satoh et al., 2013). SATB1 is also regulated in the context of peripheral T cell activation and differentiation (Cai et al., 2006; Lund et al., 2005), with increased expression required for Th2 differentiation and cytokine gene expression (Cai et al., 2006; Ahlfors et al., 2010; Notani et al., 2010). Conversely, reductions in SATB1 expression are critical for regulatory T cell function (Beyer et al., 2011).

Given the well-established role of chromosome architecture in regulating gene expression and assembly of antigen receptor loci (Jhunjhunwala et al., 2009; Shih and Krangel, 2013), we investigated whether SATB1 functions to regulate V(D)J recombination in DP thymocytes. We found that \(Tcr\) gene rearrangement is partially impaired in SATB1-deficient thymocytes, a defect that was associated with substantially reduced expression of \(Rag1\) and \(Rag2\) at the DP stage. Our analysis of this expression defect revealed that the ASE and \(Rag\) promoters interact over long-distances in DP thymocytes and that SATB1 is important to bring \(Rag2\) into this complex and to load RNA polymerase II (RNA pol II) to the \(Rag1\) and \(Rag2\) promoters. Our results provide a novel framework for understanding ASE function and the mechanistic basis for \(Rag\) gene expression in DP thymocytes.

**RESULTS**

**Impaired Tera rearrangement in SATB1−deficient DP thymocytes**

To investigate its role in V(D)J recombination, we disrupted the gene encoding SATB1 in long-term hematopoietic stem cells of \(Satb1^{f/f}\) mice (unpublished data) using a \(V\alpha\-Cre\) transgene. Unlike \(Satb1\)-null mice (Alvarez et al., 2000), \(Satb1^{f/f}\)/\(V\alpha\-Cre\) mice displayed normal growth and survival. Total thymocyte numbers were reduced \(\sim60\%\) in \(Satb1^{f/f}\)/\(V\alpha\-Cre\) as compared with \(Satb1^{f/f}\) (also referred to as WT) mice, with similar reductions in the numbers of DN and DP thymocytes (Table 1 and Fig. S1). However, there were 85–90% reductions in SP thymocytes. Thus, \(Satb1\)-deficient thymocytes undergo a normal DN to DP transition, but an impaired transition from DP to SP. \(Satb1\) mRNA expression was reduced by \(\sim95\%\) in DN3 thymocytes from \(Satb1^{f/f}\)/\(V\alpha\-Cre\) mice and its expression was essentially undetectable in DP thymocytes (Table 2).

We tested for V(D)J recombination defects using PCR to quantify \(Tcr\) locus \(V\alpha\-to-\(J\alpha\) rearrangement in genomic DNA from WT and \(Satb1^{f/f}\)/\(V\alpha\-Cre\) thymocytes. The murine \(TcrA/TcrD\) locus contains \(\sim100\) \(V\alpha\ and 61 \(J\alpha\ gene segments which can undergo several rounds of recombination in DP thymocytes (Krangel, 2009). Newly generated DP thymocytes preferentially rearrange \(V\alpha\ segments to the most 5′ \(J\alpha\
segments, whereas more mature DP thymocytes may replace these initial rearrangements by joining upstream Vα to more 3′ Jα segments. In Satb1fl/flVav-Cre thymocytes, quantitative PCR revealed relatively normal Vα rearrangement to 5′ Jα segments, but impaired rearrangement to 3′ Jα segments (Fig. 1A). This represented a deficiency in RAG-mediated cleavage at 3′ Jα gene segments rather than a deficiency in double-strand break repair, because similar reductions in signal end recombination intermediates at 3′ Jα gene segments were detected by ligation-mediated PCR (Fig. 1B).

Defective 3′ Jα usage can result from impaired thymocyte survival (Guo et al., 2002). However, the survival of purified Satb1fl/flVav-Cre DP thymocytes in culture was reduced only slightly as compared with WT thymocytes (Fig. 1C). To further investigate a role for DP thymocyte viability in the Jα rearrangement defect, we introduced a Bcl2 transgene into the Satb1fl/flVav-Cre and WT backgrounds to extend thymocyte lifespan (Fig. 1D). We then analyzed equal amounts of Tna cDNA for 5′ and 3′ Jα gene segment usage. Consistent with the rearrangement defect noted above (Fig. 1A and B), we observed a strong bias toward 5′ Jα usage in Satb1fl/flVav-Cre as compared with WT thymocytes (Fig. 1D; note that 5′ Jα usage in Satb1fl/flVav-Cre thymocytes appears to be elevated over WT because Jα signals were normalized to total Tna transcripts in this assay). The Bcl2 transgene had minimal effect on the pattern of Jα usage in WT thymocytes; the notable exception was Jα2, where Bcl2 expression appeared to protect from cell death those thymocytes that had exhausted the entire Jα array but failed to be positively selected. Although the Bcl2 transgene partially ameliorated the strong bias toward 5′ Jα usage in Satb1fl/flVav-Cre thymocytes, the 5′ bias nevertheless persisted and Jα usage in Satb1fl/flVav-Cre Bcl2tg and Satb1fl/fl Bcl2tg thymocytes remained distinct. Thus, differential lifespan of DP thymocytes cannot account for differential Jα usage in SATB1-deficient and WT thymocytes. We also detected no change in germline transcription across the Jα-Cα region, suggesting that differential Jα usage does not reflect a change in Jα locus accessibility (Fig. 1E).

**Reduced Rag1 and Rag2 gene expression in SATB1-deficient DP thymocytes**

Previous work had shown that impaired 3′ Jα usage can result from defective Rag gene expression in DP thymocytes (Yannoutsos et al., 2001). We therefore assessed Rag1 and Rag2 mRNA expression in DN and DP thymocytes from Satb1fl/flVav-Cre and WT mice using real-time PCR (Fig. 2A). Consistent with previous results (Yannoutsos et al., 2004) Rag1 and Rag2 expression were higher in DP thymocytes than in DN3 thymocytes from WT mice. SATB1-deficient thymocytes displayed normal Rag1 and Rag2 expression at the DN stage but displayed ~70 and 80% reductions in Rag1 and Rag2 expression, respectively, in DP thymocytes. Similar reductions in Rag1 and Rag2 transcripts were observed in DP thymocytes from Satb1fl/flLck-Cre mice; thus,

### Table 1. Quantification of thymocyte subsets in WT (Satb1fl/fl) and Satb1−/− (Satb1fl/flVav-Cre) mice

| Cell population       | WT (×10⁶)      | Satb1−/− (×10⁶) |
|-----------------------|----------------|-----------------|
| Total thymocytes (n = 6-7) | 1.730 ± 610 | 670 ± 300³     |
| DN1 (n = 3-4)         | 0.45 ± 0.30   | 0.20 ± 0.17     |
| DN2 (n = 3-4)         | 0.89 ± 0.10   | 0.11 ± 0.10e   |
| DN3 (n = 3-4)         | 17.9 ± 10.3   | 5.1 ± 3.4      |
| CD8 ISP (n = 5-6)     | 22.0 ± 11.3   | 12.4 ± 9.1     |
| DP (n = 6-7)          | 1,460 ± 500   | 570 ± 250⁰     |
| CD4 SP (n = 6-7)      | 109 ± 49      | 17 ± 2²⁰       |
| CD8 SP (n = 6-7)      | 28.2 ± 11.6   | 2.7 ± 2.1³    |

*Vav-Cre transgenic mice have no intrinsic thymic phenotype (de Boer et al., 2003).
²P < 0.01 when compared with WT counterparts (Student’s t test).
³P < 0.001 when compared with WT counterparts (Student’s t test).

### Table 2. Satb1 mRNA expression (relative to B2m) in thymocyte subsets of WT (Satb1fl/fl) and Satb1−/− (Satb1fl/flVav-Cre) mice

| SATB1/β2–microglobulin | WT (×10⁴) | Satb1−/− (×10⁴) |
|-------------------------|-----------|-----------------|
| DN3 (n = 2)             | 3.28 ± 0.31 | 0.19 ± 0.02     |
| DN4 (n = 2)             | 36.5 ± 2.68 | 0.13 ± 0.005    |
| CD8 ISP (n = 2)         | 90.9 ± 40.8 | ND⁴             |
| DP (n = 2)              | 241 ± 51.5  | 0.0046 ± 0.0008 |
| CD4 SP (n = 2)          | 26.4 ± 3.49 | 0.093 ± 0.041   |
| CD8 SP (n = 2)          | 5.15 ± 0.30 | 0.0049 ± 0.0029 |

*RNA and cDNA were prepared from 30,000 cells.
⁴ND, not detected.
SATB1 regulates thymocyte Rag gene expression | Hao et al.

The Rag expression defect is T cell intrinsic (Fig. 2 B). Consistent with the reduced expression phenotype, chromatin immunoprecipitation (ChIP) revealed substantial reductions in the transcription-associated modification histone H3 lysine 4 trimethylation (H3K4me3) at the Rag1 and Rag2 promoters and gene bodies in Satb1<sup>−/−</sup>Vav-Cre DP thymocytes (sites L, M, O and P, Fig. 2 C). We conclude that biased 5′ Jα usage in Satb1<sup>−/−</sup>Vav-Cre thymocytes reflects inefficient
Figure 2. Defective \textit{Rag1} and \textit{Rag2} gene expression in SATB1-deficient and ASE-deleted DP thymocytes. (A) The abundance of \textit{Rag1} (left) and \textit{Rag2} (right) transcripts in CD25$^+$ (DN2+3) and DP thymocytes was evaluated by quantitative RT-PCR. The data represent the mean ± SEM of four experiments for DN2+3 and five experiments for DP (WT, \textit{Satb1}^+/−; \textit{Satb1}^−−, \textit{Satb1}^+/−\textit{Vav-Cre}; one mouse per genotype per experiment) with normalization to values for \textit{Actb}. *, P ≤ 0.05 by two-tailed Student’s t test. (B) A T cell–intrinsic effect of SATB1 was evaluated by measuring transcript abundance in DP thymocytes of WT (\textit{Satb1}^+/+) and \textit{Satb1}^+/−\textit{Lck-Cre} mice by quantitative RT-PCR. The data represent mean ± SEM of three experiments for WT and four experiments for \textit{Satb1}^+/−\textit{Lck-Cre} cDNA (one mouse per experiment), with normalization to values for \textit{hprt}. *, P ≤ 0.05 by two-tailed Student’s t test. (C) ChIP analysis of \textit{Rag} locus histone H3K4me3. A map of the \textit{Rag} locus depicts the convergently transcribed \textit{Rag1} and \textit{Rag2} genes and known cis-regulatory elements ASE, \textit{Erag} (enhancer of \textit{Rag}), \textit{D3} (distal enhancer), \textit{Ep} (enhancer proximal), and \textit{Sil} (silencer; Kuo and Schlissel, 2009). Sorted DP thymocytes of WT and SATB1-deficient mice were analyzed by ChIP followed by quantitative PCR. Sites A–R in the \textit{Rag} locus were analyzed by ChIP. The \textit{MageA2} promoter (\textit{Mg}) served as a negative control. The data represent the mean ± SEM of three experiments for each genotype (one mouse per experiment), with values of bound/input expressed relative to those for the \textit{B2m} promoter (normalized to 1) in each sample. *, P ≤ 0.05 by two-way ANOVA with Sidak’s multiple comparisons test. (D) \textit{Tcra} coding joints in thymocytes of ASE-deleted mice. Thymocyte genomic DNA samples were amplified by quantitative PCR using a \textit{Vq} family primer in conjunction with different \textit{Jq} primers, with values for ASE−− thymocytes normalized to those for WT littermates. The data represent the mean ± SEM of 2–3 independent preparations for each genotype. *, P ≤ 0.05 by two-tailed Student’s t test. (E) ChIP analysis of \textit{Rag} locus histone H3K4me3 in DP thymocytes of ASE−− mice. The data represent the mean ± SEM of four experiments for WT (ASE+/+; one mouse per experiment) and five experiments for ASE−− (one mouse per experiment). P ≤ 0.05 by two-way ANOVA with Sidak’s multiple comparisons test.

rearrangement due to reduced \textit{Rag} gene expression, and that this 5' bias may be partially ameliorated by the \textit{Bcl2} tg (Fig. 1 D) because thymocytes have additional time for rearrangements to occur.

\textbf{Stage-specific and SATB1-dependent conformations of the \textit{Rag} locus}

Because \textit{Satb1}^{f+/−}\textit{Vav-Cre} mice displayed a DP stage-specific defect in \textit{Rag} gene expression, we hypothesized that SATB1
SATB1 regulates thymocyte Rag gene expression | Hao et al.

might play a role in ASE function, perhaps by facilitating interactions between the ASE, 71-kb upstream of Rag2, and more proximal cis-acting elements. Prior analysis of ASE−/− mice revealed Rag1 and Rag2 gene expression to be <1% of the level in WT DP thymocytes (Yannoutsos et al., 2004), suggesting a more dramatic expression defect than in Satb1−/−Vav-Cre mice. In accord with this, we found that ASE−/− DP thymocytes displayed more dramatic reductions in Vα-to-Jα recombination (Fig. 2 D) and in histone H3 lysine 4 trimethylation (H3K4me3) (Fig. 2 E) than in Satb1FcreVav-Cre mice. On this basis, we predicted that ASE functions and interactions might be partially diminished in

Figure 3. Developmentally regulated and SATB1–dependent interactions between the ASE and Rag promoters. (A) SATB1 ChIP-seq. Sequencing reads for SATB1 ChIP and input DNA are plotted. The ASE region marked corresponds to fragment A8 (see Fig. 6). Long-distance interactions of the ASE-containing HindIII (B) or BglII (C) fragments were analyzed by 3C, followed by quantitative PCR. ASE viewpoint restriction fragments are shaded dark blue and target restriction fragments are shaded light blue. Primers were all reverse orientation and were positioned at the left end of each restriction fragment. Relative cross-linking data for each restriction fragment were plotted in the center of the fragment. Data in B represent mean ± SEM of two experiments with lymph node T cells (LN-T) cells (one mouse per experiment), three experiments with DN (Lat−/−) thymocytes (one litter of five to eight mice per experiment), four experiments with WT (Satb1Flox) DP thymocytes (one mouse per experiment), and four experiments with Satb1−/− (Satb1FloxVav-Cre) DP thymocytes (one mouse per experiment), all normalized to results for a nearest neighbor fragment (=1). *, P ≤ 0.05 by two-tailed Student’s t test comparing WT to Satb1−/− DP. Data in C represent mean ± SEM of two experiments with LN-T cells (one mouse per experiment), eight experiments with WT DP thymocytes (one mouse per experiment), and six experiments with Satb1−/− DP thymocytes (one mouse per experiment), normalized as in B. *, P ≤ 0.05 by two-tailed Student’s t test comparing WT to Satb1−/− DP.
Satb1<sup>F<sup>f/f</sup> Vav-Cre mice. Consistent with such a role, ChIP-seq analysis revealed prominent SATB1 binding to the ASE, as well as to the Rag1 and Rag2 promoter regions in DP thymocytes (Fig. 3 A).

Previous studies showed that the ASE is required to counteract functions of the Rag silencer; however, the mechanistic basis for this process remained uncertain (Yannoutsos et al., 2004). In this regard, the ASE could be envisioned to promote Rag gene expression as a consequence of direct physical interaction with the silencer in DP thymocytes. Alternatively, the ASE might counteract the influence of the silencer by interacting directly with the Rag gene promoters.

To investigate Rag locus architecture, we explored interactions between the ASE and Rag genes using chromosome conformation capture (3C; Dekker et al., 2002). In this approach, long-distance interactions in nuclei are initially captured by formaldehyde cross-linking; after restriction enzyme digestion, dilution, and intermolecular ligation, interacting DNA fragments are detected by real-time PCR. Using the ASE-containing HindIII fragment as a viewpoint, in WT DP thymocytes we detected interactions with HindIII fragments carrying the Rag1 and Rag2 promoters 70–100 kb away (Fig. 3 B). Similar ASE–Rag promoter interactions were detected between the relevant BglII fragments as well (Fig. 3 C). These interactions were specific, because they were not detected in LN T cells, which do not express the Rag genes. We evaluated chromatin interactions in the DN compartment by analyzing thymocytes from LAT-deficient mice (Zhang et al., 1999). Interaction frequencies were only slightly higher than in LN T cells (Fig. 3 B), consistent with a limited role for the ASE in Rag gene expression in DN thymocytes (Yannoutsos et al., 2004).

Notably, ASE interactions with the Rag2 promoter were significantly attenuated in Satb1<sup>F<sup>f/f</sup> Vav-Cre DP thymocytes (Fig. 3, B and C). However, ASE–Rag1 promoter contacts were either maintained (Fig. 3 B) or marginally reduced in a manner that did not reach statistical significance (Fig. 3 C). To address this point further, we analyzed an independent set of BglII-digested 3C samples and confirmed reduced ASE–Rag2 promoter interaction but normal ASE–Rag1 promoter interaction in Satb1<sup>f/f</sup> Vav-Cre DP thymocytes (Fig. 4, A and B). Moreover, the requirement for SATB1 to facilitate ASE–Rag2 promoter interaction was highly specific, because Rag2 promoter contacts with the Traf6 promoter were maintained in SATB1-deficient mice (Fig. 4, A and C). Although the Rag2–Traf6 interaction serves as a useful control, it is of unknown biological significance.

Our 3C data indicated that, in addition to the Rag promoters, the ASE interacts broadly across the Rag locus (above the background in LN T cells), including sites between the ASE and Rag2, and within the Rag2 and Rag1 gene bodies (Fig. 3, B and C). Although the ASE contacts the region that includes the intergenic Rag silencer, these interactions were not elevated above the general levels observed across the Rag locus. Collectively, these data support a model in which the ASE indirectly counteracts silencer activity via direct interactions with the Rag1 and Rag2 promoters.
The ASE organizes the *Rag* locus

To further explore the role of the ASE in *Rag* locus conformation, we used 3C to assess long distance interactions in DP thymocytes from ASE^{−/−} mice. Using the *Rag1* promoter fragment as a viewpoint, we detected an interaction between the *Rag1* and *Rag2* promoters in WT DP thymocytes and found this interaction to be substantially diminished in ASE^{−/−} DP thymocytes (Fig. 5 A). Similar results were obtained using a *Rag2* promoter-containing fragment as the viewpoint (Fig. 5 B). Moreover, *Rag1*−*Rag2* promoter interactions were reduced partially in *Satb1^{f/f} Vav-Cre* DP thymocytes (Fig. 5, A and B). Using these new viewpoints, we also confirmed reduced interactions of the *Rag2* but not the *Rag1* promoter with the ASE in *Satb1^{f/f} Vav-Cre* DP thymocytes (Fig. 5, A and B), a finding consistent with 3C analyses from the ASE viewpoint (Figs. 3 and 4). We conclude that the ASE organizes the *Rag* locus by tethering the *Rag1* and *Rag2* promoters in DP thymocytes, and that efficient tethering of the *Rag2* promoter depends on SATB1.

The ASE is required for RNA pol II occupancy at the *Rag* promoters

To explore the functional significance of the DP-specific chromatin conformation of the *Rag* locus, we analyzed the RNA pol II distribution using ChIP. As anticipated, RNA pol II occupancy was high at the *Rag1* and *Rag2* promoters in WT DP thymocytes (sites L and Q, Fig. 6 A). However, RNA pol II was prominent at the ASE and silencer as well (sites C and N, Fig. 6 A). In DN thymocytes from *Lat^{−/−}* mice, lower levels of RNA pol II were present at the *Rag* promoters and silencer, but at the ASE, RNA pol II was as abundant as in DP thymocytes. In contrast, RNA pol II was minimal at all sites in LN T cells (Fig. 6 A) and in ASE^{−/−} DP thymocytes (Fig. 6 B). Thus, RNA pol II occupancy at the *Rag* promoters and silencer is developmentally regulated in a manner that correlates with transcription and depends on the ASE. A previous study also identified substantial RNA pol II binding to the *Rag* promoters, ASE, and silencer in DP thymocytes, although other stages of T cell development were not examined (Koch et al., 2011).
Notably, RNA pol II occupancy at the *Rag* promoters and silencer was partially reduced in DP thymocytes from *Satb1*^−/−^ (Satb1^f/f^Vav-Cre) mice, whereas occupancy at the ASE was unaffected (Fig. 6 C). Thus, RNA pol II occupancy at the ASE occurs independent of ASE–promoter interactions, whereas RNA pol II occupancy at the promoters and silencer correlates with assembly of an intact complex containing the ASE and the *Rag1* and *Rag2* promoters. Similarly, DN thymocytes display RNA pol II at the ASE but, in the absence of ASE–promoter interactions, only low levels at the *Rag* genes themselves (Fig. 6 A). We suggest that the *Rag1* and *Rag2* promoters acquire high levels of RNA pol II when they are tethered to

![Figure 6. Locus conformation-dependent RNA pol II occupancy of the *Rag1* and *Rag2* promoters.](image-url)
The ASE directly activates the Rag1 and Rag2 promoters
The above data imply that the ASE stimulates Rag gene transcrip-
tion through direct long distance interactions with the Rag1 and Rag2 promoters, perhaps functioning as a classical enhancer. To test this possibility, we sought to define an ASE fragment that activates Rag1 and Rag2 promoter-driven luciferase reporter constructs in transiently transfected cells. Because previous work had only ascribed ASE activity to a broad 8-kb region (Yannoutsos et al., 2004), we asked whether a smaller core ASE possessed functional activity. Initially, we leveraged chromatin data from the mouse ENCODE database (Stamatoyannopoulos et al., 2012; Rosenbloom et al., 2013) to narrow our search. DNase-Seq data from unsorted thymocytes reveals a tightly linked pair of strong DNase I hypersensitive sites that span ~2-kb and correspond to a previously identified region notable for substantial interspecies sequence conservation (Fig. 7 A; Yannoutsos et al., 2004). We further mapped open chromatin in the Rag locus in purified DP thymocytes using formaldehyde-assisted isolation of regulatory elements (FAIRE; Giresi et al., 2007), which similarly detected a 2-kb open chromatin region (Fig. 7 B). ENCODE data show that the same region harbors the characteristic chromatin signature of active enhancers in thymus but not in spleen: high histone H3 lysine 4 monomethylation (H3K4me1) and histone H3 lysine 27 acetylation (H3K27ac) coupled with low histone H3 lysine 4 trimethylation (H3K4me3) and histone H3 lysine 27 trimethylation (Fig. 7 A; Bulger and Groudine, 2011; Natoli and Andrau, 2012; Calo and Wysocka, 2013). Moreover, our ChIP-seq data mapped SATB1 binding to the same region and, consistent with our ChIP data (Fig. 6 A), the region displayed high RNA pol II occupancy as well (Fig. 7 A).

We tested enhancer activity of the 2.0-kb ASE fragment that corresponded to the region of DNase I hypersensitivity and sequence conservation, as well as a 1.3-kb subfragment (AB) corresponding to the region of highest H3K4me1, H3K27ac, and RNA pol II. These test fragments were introduced into promoter-driven luciferase reporters that were transiently transfected into the murine DP thymocyte cell line VL3-3M2 (Fig. 7 C). By themselves, the Rag1 and Rag2 promoters drove minimal luciferase expression compared with positive control plasmids containing the promoters plus the Tαα enhancer (Eαα), a potent enhancer in DP thymocytes. Notably, the 2-kb ASE fragment and subfragment AB were more potent than Eαα as activators of both promoters. To determine the minimal region responsible for enhancer activity, we tested two partially overlapping subfragments of AB (fragments A and B). Both displayed enhancer activity, with the activity of the 2-kb ASE or fragment AB only marginally better than either A or B. This suggested that the 140-bp region shared by fragments A and B, which is centered within the strong peaks of H3K4me1, H3K27ac, RNA pol II, and SATB1, might play a dominant role in enhancer activity. Indeed, fragments A’ and B’, truncated versions of A and B lacking the 140-bp overlap region, displayed no detectable enhancer activity, whereas the 140-bp overlap region exhibited enhancer function comparable to that of Eαα. We conclude that the 140-bp ASE fragment, which contains evolutionarily conserved binding sites for E2A-, Runx-, GATA-, and Ikaros-family transcription factors (Fig. 7 D), is a core enhancer element that augments the function of the Rag promoters. However, because activity of the 140-bp fragment was substantially reduced relative to larger ASE fragments, flanking sequences are likely to boost enhancer activity even further.

DISCUSSION
The Rag silencer and ASE were previously shown to coordinate Rag gene expression in DP thymocytes (Yannoutsos et al., 2004). The silencer was defined using bacterial artificial chromosome (BAC) reporter transgenes lacking the ASE. In such constructs, silencer deletion caused increases in Rag gene expression in DN and DP thymocytes but not in developing B cells. Moreover, the silencer could suppress the expression of a heterologous reporter transgene in pre–B cells, DN and DP thymocytes, and splenic T cells. Thus, the silencer appeared to be capable of broadly suppressing the activities of linked promoters in lymphoid cells. In BAC constructs containing the intergenic silencer, the ASE was shown to be required for Rag expression in DP thymocytes and to increase Rag expression in DN3 thymocytes. However, it had no apparent effect on Rag expression in constructs lacking the silencer. Consistent with these observations, gene-targeted deletion of the ASE reduced Rag expression in DN thymocytes, abrogated Rag expression in DP thymocytes, and caused a developmental block at the DP stage that was likely secondary to impaired Tia1 gene recombination. Thus, ASE activity was strongest in DP thymocytes, but the ASE was judged to be distinct from a classical enhancer because it appeared to function by counteracting the activity of an intergenic silencer (Yannoutsos et al., 2004).

These foundational studies left the mechanistic basis for ASE activity unclear. As one possibility, the ASE could functionally interact with the silencer to neutralize its ability to suppress the Rag promoters. Alternatively, the ASE could functionally interact with the Rag promoters to overcome the suppressive effects of the silencer. Our data strongly support the latter scenario and argue that the ASE functions as a classical enhancer: it displays a chromatin signature typical of active enhancers, it interacts physically with the distant Rag1 and Rag2 promoters and brings these promoters together to form a chromatin hub in DP thymocytes (Fig. 8), and it can directly and potently activate the Rag1 and Rag2 promoters in the absence of the silencer or any other locus elements.

Our data yield a picture that diverges in two respects from the initial description of ASE activity. First, the chromosomal BAC reporter studies identified no ASE activity in constructs lacking the silencer (Yannoutsos et al., 2004), whereas we show that the ASE can function as a direct activator of the Rag1 and Rag2 promoters in extrachromosomal luciferase
Figure 7. The ASE directly activates the *Rag1* and *Rag2* promoters. (A) ENCODE data tracks in the Mouse July 2007 (NCBI37/mm9) assembly are shown. (top) DNase-seq data for the *Rag* locus in unfractionated thymocytes. (bottom) ChIP-Seq data and sequence conservation for the DNase hypersensitive region in unfractionated thymocytes and splenocytes. Note that primer sets B and D (Fig. 6) map upstream and downstream, respectively, of the 2-kb open chromatin region whereas primer set C maps within the 140-bp minimal enhancer region. (B) FAIRE samples were prepared to evaluate open chromatin at the ASE in WT DP thymocytes. Quantitative PCR was used to evaluate enrichment of sequences in DNA purified from formaldehyde-cross-linked as compared with uncrosslinked samples. The *Tcra* enhancer (Ex) and MageA2 (Mg) served as positive and negative controls, respectively. Positions of test amplicons relative to the 8-kb ASE region are indicated above the graph. The data are presented as mean ± SEM of values of crosslinked/uncrosslinked in three experiments (one mouse per experiment). *, *P ≤ 0.05 by two-tailed Student's *t* test comparing Ex and test amplicons to MageA2. (C) ASE activation of the *Rag1* and *Rag2* promoters. Test ASE fragments were cloned downstream of either a *Rag1* promoter-driven or a *Rag2* promoter-driven luciferase gene and were assayed by transient transfection into VL3-3M2 DP thymocytes. The data represent the mean ± SEM of two to four independent experiments. *, *P ≤ 0.05 by two-tailed Student's *t* test comparing results for test ASE fragments to promoter-only controls (−). (D) Nucleotide sequence of the 140-bp minimal enhancer with predicted binding sites for transcription factors marked.
reports that lack the silencer. One explanation for this difference could be that the analysis of a small number of BAC integrants did not provide an accurate picture of ASE activity. However, we favor an alternate possibility: in the environment of a chromosomally integrated locus, the silencer may be essential to create a repressive environment at the promoters that would enforce the need for ASE activity, whereas extrachromosomal reporters may be intrinsically suppressed at the promoters even in the absence of the silencer. A second point of divergence is that the BAC reporter studies showed the ASE to be active but not essential for Rag gene expression in DN thymocytes, whereas we see no clear evidence for long-distance interaction between the ASE and the Rag promoters in that compartment. This difference may simply reflect insensitivity of our 3C analysis to low frequency ASE–promoter interactions that may occur in DN thymocytes. Nevertheless, the BAC transgene and our current work are concordant in many regards, especially the greater reliance on ASE activity and long-distance interactions in DP compared with DN thymocytes.

The intriguing distribution of RNA pol II at the Rag locus suggests potential mechanisms by which the ASE might regulate Rag gene expression. At the ASE, we found RNA pol II to be present at high levels in DN and DP thymocytes and in SATB1-deficient DP thymocytes, but not in LN T cells. However, high level RNA pol II occupancy at the Rag1 and Rag2 promoters was only detected in SATB1-sufficient DP thymocytes, thereby correlating with looping between the ASE and the two Rag promoters. This suggests that an important function of these regulatory loops is to promote RNA pol II loading to the promoters (Fig. 8). RNA pol II binding is a shared property of many enhancers and is often associated with the transcription of enhancer RNAs (Kim et al., 2010; Koch et al., 2011; Natoli and Andrau, 2012). Recent data suggest that enhancer RNAs may play a direct role in enhancer-promoter looping and promoter activation via a mechanism that may involve cohesin (Li et al., 2013). Consistent with these possibilities, the ASE binds TATA–binding protein and general transcription factors in addition to RNA pol II (Koch et al., 2011), and there is low-level ASE transcription in DP thymocytes. RNA pol II initially recruited to the ASE could be delivered to the Rag promoters as a consequence of ASE–promoter interactions. Alternatively, ASE–promoter interactions could stabilize the binding of newly recruited RNA pol II at the Rag promoters. Viewed in another way, the ASE may frequently localize to a transcription factor (Cisse et al., 2013; Cook, 1999; Ghamari et al., 2013) in DN and DP thymocytes, and ASE–promoter interactions and elevated promoter RNA pol II occupancy may reflect high frequency recruitment of the Rag promoters into the same transcription factory in DP thymocytes. The substantially reduced Rag transcription that occurs in DN thymocytes may then be interpreted as a reduced frequency of promoter recruitment to ASE-containing transcription factories in these cells.

RNA pol II was also detected in the Rag silencer region. This binding had a profile similar to that of the Rag promoters, in that it was elevated in DP thymocytes in an ASE- and SATB1-dependent manner, thereby correlating with long-distance looping and high levels of Rag gene transcription. Of note, RNA pol II occupancy maps to the very 3′ end of the Rag1 transcription unit (Rosenbloom et al., 2013; Stamatoyannopoulos et al., 2012), ~700 bp away from the Runx binding-site that was shown to be essential for silencer activity (Yannoutsos et al., 2004). Moreover, this RNA pol II is primarily in the serine 2 phosphorylated form (Koch et al., 2011). Based on this, we suspect that the accumulation of RNA pol II at this site is related to the termination of Rag1 transcription and not to silencer function.

Finally, our data suggest important roles for chromatin organizer SATB1 in Rag gene expression and Tera recombination in DP thymocytes. SATB1 expression is dramatically up-regulated in DP thymocytes (Table 2), correlating with the substantial increases in Rag gene expression in this compartment. Further, SATB1 binds to multiple sites across the Rag locus, most prominently at the ASE and Rag1 and Rag2 promoters. We suspect that SATB1 binding to the Rag locus is up-regulated in parallel with its increased expression in DP thymocytes, although our experiments do not directly address this point. However, loss of SATB1 led to significant reductions in Rag gene expression, in Rag2 promoter interactions with the ASE and Rag1 promoter, and in RNA pol II occupancy at the Rag promoters and silencer in DP thymocytes.
Flow cytometry and cell sorting. For surface staining, erythrocyte-lysed single-cell suspensions were prepared in staining medium (DMEM + 2% FCS) with 1 mg/ml normal rat IgG (Sigma-Aldrich) and were stained on ice for 20 min using the following antibodies: FITC–CD8α (53–6.7), PE–CD3ε (145–2C11), PE–Cy5–B220 (RA3–6B2), Mac-1 (M1/70), Gr-1 (RB6–8C5), TER119 (TER-119), PE–Cy7–CD4 (GK1.5), APC–CD44 (IM7), TCR (145–2C11), PE–Cy5–B220 (RA3–6B2), Mac-1 (M1/70), Gr-1 (RB6–8C5), and PE–Cy5–CD11c (HL3) from BD. FACS analyses were performed on a FACS Canto II (BD) and data were analyzed with FlowJo software (Tree Star).

For cell sorting, single-cell suspensions were prepared following erythrocyte lysis. DN3 thymocytes (CD4⁺ CD8⁻ CD25⁺ CD44⁻) and a combined DN2/3 thymocyte population (CD4⁺ CD8⁺ CD25⁻) were obtained after initial depletion of CD4⁺ and CD8⁺ cells using biotinylated-CD4 and CD8 antibodies (GK1.5 and 53–6.7, respectively) (eBioscience) and streptavidin MACS beads (Miltenyi Biotec). DP thymocytes (CD4⁺ CD8⁺) were obtained by sorting of whole thymocytes using FITC–CD8 (53–6.7) and PE–Cy7–CD4 (GK1.5) antibodies (eBioscience) or PE–CD4 (GK1.5) and FITC–CD8 (53–6.7) antibodies (BioLegend). Sorting was performed on a FACSVantage (BD) and the purity of cells after double sorting was >95%. Lymph node T cells were isolated as previously described (Jackson and Krangel, 2005).

In vitro survival assay. Sorted DP thymocytes (10⁵) were cultured in 200 µl RPMI 1640 medium containing 10% FBS, 55 µM 2-mercaptoethanol, 2 mM l-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin for varying times in the presence of 10 ng/ml mouse recombinant IL-7 (R&D Systems). Apoptosis was measured by Annexin V and 7-aminocoumarin D staining using the Annexin V-PE kit (BD) according to the manufacturer's instructions.

ChIP. For immunoprecipitation using anti-trimethylated H3K4 (Millipore 04–74) or control rabbit IgG (ab-105-c; R&D Systems), chromatin was prepared without formaldehyde cross-linking and was immunoprecipitated exactly as previously described (Hao and Krangel, 2011). Immunoprecipitated and input samples were quantified by real-time PCR using a Roche LightCycler and a FastStart DNA Master Syber Green I kit (Roche). PCR conditions were as follows: 5 min at 95°C followed by 45 cycles of 1 s at 95°C, 5 s at 62°C, 7 s at 72°C. Analysis of ΔΔCt was used to normalize ratios of bound/input in different samples. Primers sequences are provided in Table S1.

For immunoprecipitation using anti-RNA pol II (Millipore; 05–623) or control rabbit IgG (R&D Systems; ab-105-c), 1 × 10⁶ cells were subjected to cross-linking by incubation for 10 min on ice in 10 ml of RPMI 1640 containing 10% FBS and 1% formaldehyde. The reaction was stopped by addition of glycine to 0.125 M and incubation for 5 min at 23°C. Cells were then washed in PBS and lysed by incubation for 10 min on ice in 1 ml of 5 mM PIPES, pH 8.0, 85 mM KCI, 0.5% NP-40, 0.1 mM PMSF, and 0.1 mM benzamidine. Nuclei were pelleted and lysed by resuspension in 0.3 M 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 1% SDS. Chromatin was then pelleted through 8 ml urea by centrifugation at 30,000 rpm for 16 h at 10°C in a Beckman SW 40 Ti rotor. After centrifugation, pelleted chromatin was resuspended in 10 mM Tris, pH 8.0, 1 mM EDTA, 5% glycerol, was dialyzed overnight at 4°C against the same buffer. The volume was then adjusted to 1 ml and the suspension was sonicated using a Model 580 Sonic Dismembrator (Thermo Fisher Scientific), alternating 15 s on and 20 s off for 10 cycles with the sample immersed in an ice/water bath. Chromosomal DNA was reduced to an average size of 300–500 bp, as determined by agarose gel electrophoresis. Sonicated chromatin was precloned with Protein A-Sepharose/salmon sperm DNA slurry (Millipore), incubated overnight at 4°C with anti-RNA pol II or control rabbit IgG, and was subsequently incubated for 1 h with Protein A-Sepharose/salmon sperm DNA slurry. Immunoprecipitated DNA was then purified after rigorous washing of immunoprecipitates and reversal of cross-links by overnight incubation at 60°C. Immunoprecipitated and input samples were quantified by real-time PCR using a Roche LightCycler 480 and a QuantFast SYBR green kit (QIAGEN). PCR conditions were as follows: 5 min at 95°C followed by 45 cycles of 10 s at 95°C, 30 s at 62°C.

ChIP-seq. For ChIP-seq using anti-SATB1 (Abcam; ab109122), 4 × 10⁷ C57Bl6/6 thymocytes were freshly prepared and washed with PBS containing 0.5 mM PMSF and were subjected to cross-linking by incubation for 10 min in 5 mM Heps, pH 7.5, 10 mM NaCl, 0.1 mM EDTA, 0.05 mM EGTA, 1% formaldehyde at 23°C. The reaction was stopped by addition of glycine to 0.125 M. Cells were then immediately washed in ice-cold PBS containing 0.5 mM PMSF and lysed by incubation for 10 min on ice in 1 ml of 50 mM Heps, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% Glycerol, 0.5% NP-40, 0.25% TritonX-100 containing protease inhibitor (Roche). Nuclei were pelleted and lysed by resuspending in 10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, and 0.5 mM EGTA containing protease inhibitor (Roche). Pelleted chromatin was resuspended in 400 µl of 10 mM Tris, pH 8.0, 300 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% TritonX-100, 0.1% sodium deoxycholate and 0.5% N-laurylsarcosine, and was sonicated using a model XL2000 ultrasonic cell disruptor (MICROSON) so that relatively large fragments (1–10 kb) were included. Sonicated chromatin was incubated overnight at 4°C with anti-SATB1 antibody that was preconjugated (BioLegend). Sorting was performed on a FACSVantage (BD) and the purity of cells after double sorting was >95%. Lymph node T cells were isolated as previously described (Jackson and Krangel, 2005).
with Magnet beads (Dynabeads). Immunoprecipitated DNA was then purified after vigorous washing of immunoprecipitates by the sequential addition of cross-links by overnight incubation at 65°C in 50 mM Tris, pH 8.0, 10 mM EDTA, and 1% SDS. Input DNA and ChIP DNA (10–20 ng) were subjected to sonication (Bioruptor; UCSD-200) at medium power for 20 min, alternating 30 s on and 30 s off. Library construction was conducted as follows: (1) End repair by T4 DNA polymerase, Klenow DNA polymerase, and T4 kinase; (2) A-tailing by Klenow exo-; (3) Adapter ligation using Illumina TruSeq adapters (Illimina) and T4 DNA ligase; (4) 15 cycles of PCR amplification by AccuPrime DNA Taq polymerase (Illuminio) and TruSeq primers; (5) purification of final products by size selection (200–1,000 bp) using SPR1 beads (Thermo Fisher Scientific). The ChIP-sequencing was performed by the UC Berkeley sequence facility. The Gene Expression Omnibus accession no. for the SATB1 ChIP-seq dataset is GSE66248.

Quantitative RT-PCR. Total RNA was isolated using TRIzol (Illuminio) according to the manufacturer’s instructions, and cDNA was synthesized using oligo dT primers and SuperScript III (Illuminio). PCR was performed using an iCyte MyQ Real-Time PCR Detection System (Bio-Rad Laboratoyes) and SYBR Green PCR core reagents (Applied Biosystems). PCR conditions (for Rag1, Rag2, and Actb) were as follows: 95°C for 10 min, followed by 25 cycles of 94°C for 30 s, 62°C for 30 s and 72°C for 1 min, and a 10-min extension at 72°C. Amplicons were separated by agarose gel electrophoresis and analyzed by Southern blot for the SATB1 ChIP-seq dataset is GSE66248.

3C. This technique was performed with some modifications to a previously described protocol (Hajjige et al., 2007). 1 × 10^6 cells were subjected to cross-linking by incubation for 10 min on ice in 10 µl of RPMI-1640 containing 10% FBS and 2% formaldehyde. The reaction was stopped by addition of glycine to 0.125 M and incubation for 5 min at 23°C. Cells were then washed in PBS and lysed by incubation for 10 min on ice in 5 ml of 0.1 M Tris, pH 8.0, 10 mM NaCl, 0.2% NP-40, 0.1 mM benzamidine, and 0.1 mM PMSF. Nuclei were pelleted, washed once with PBS, and lysed by incubation for 1 h at 37°C in 0.5 ml of 1% NEB II or NEB III digestion buffer (New England Biolabs) containing 0.3% SDS. Triton-X100 was then added to a final concentration of 2% and incubation was continued for an additional 1 h at 37°C. Chromatin was then digested by addition of 200 U BglII or HindIII for overnight incubation at 37°C, followed by a second addition of 200 U of enzyme for an additional 8 h incubation at 37°C. Digested chromatin was then sedimented through 8 M urea by centrifugation at 35,000 rpm for 16 h at 10°C in a Beckman SW 40Ti rotor. After centrifugation, the digested chromatin (0.5 ml of gel and liquid) was diluted and repurified in 2 ml H2O, dialyzed overnight at 4°C against 30 mM Tris, pH 7.4, 10 mM MgCl2, diluted to 7 ml in 30 mM Tris, pH 7.4, 10 mM MgCl2, 1 mM DTT, and 0.1 mM ATP, and was ligated by addition of 4,000 U T4 DNA ligase for overnight incubation at 16°C. Ligated chromatin was incubated overnight at 60°C to reverse cross-links and DNA was purified by extraction with phenol/chloroform and ethanol precipitation.

Ligation products were quantified by TaqMan quantitative real-time PCR (Roche) using a Roche LightCycler 480 and PCR, conditions as follows: 5 min at 95°C followed by 45 cycles of 10 s at 95°C and 30 s at 62°C. To generate ligation product standards, 10 µg of BAC RP23-32513 was digested at 37°C overnight with 50 U HindIII or BglII, after which DNA was purified by phenol/chloroform extraction and ethanol precipitation, and then ligated overnight at 16°C in a 200 µl reaction containing 40 U T4 DNA ligase. Purified, ligated DNA was serially diluted in 10-fold increments from a 2 ng/µl stock to generate the standard curve.

Digestion efficiencies of different experimental samples were 90–94% as determined by quantitative real-time PCR in which yields of several amplicons that span HindIII or BglII sites were compared with yields of neighboring amplicons that were not disrupted by digestion. Normalization of 3C PCR signals from different samples was accomplished by setting the nonspecific interaction of the bait fragment with one of its nearest neighbor fragments equal to one in each sample. Primer and probe sequences are provided in Table S1.

FAIRE. FAIRE was performed essentially as previously described (Giresi et al., 2007) except that quantitative real-time PCR was used to evaluate enrichment of sequences in DNA purified from formaldehyde-cross-linked as compared with uncrosslinked samples. Primer sequences are provided in Table S1.

Luciferase. VL3-3M2 cells were provided by S. Sarafova (Davidson College, Davidson, NC) and were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 55 µM 2-mercaptoethanol, 2 mM t-glutamine, and 25 mM Hepes, pH 7.0. Approximately 3 × 10^5 cells were transfected with versions of the pXPG firefly luciferase reporter plasmid (Bert et al., 2000) containing the Rag1 or Rag2 promoter and different anti-silencer region or control DNA fragments. In brief, 1 µg of each construct was cotransfected with 100 ng of plasmid expressing Renilla luciferase using the Superfect transfection reagent (QIAGEN). Cells were cultured for 48 h in 0.5 ml of medium in a 24-well plate and were then harvested to assay for luciferase activity using an Infinite F200 Tecan plate luminometer and a Dual-Luciferase Reporter Assay kit (Promega) according to the manufacturer’s instructions.

Online supplemental material. Fig. S1 shows normal DN-to-DP transition in SATB1-deficient thyrocytes. Table S1 lists oligonucleotides used as PCR primers and probes. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20142207/DC1.

We thank Lynn Martinez, Nancy Martin, and Mike Cook of the Duke Cancer Institute Flow Cytometry Facility for help with cell sorting and analysis, Kingshuk Roy Chowdhury for advice on statistical analysis, and Eugene Oltz for critical reading of the manuscript.

References

Ahlfors, H. A., Linzey, I. L., Elo, S. Tuomela, M. Burute, K. V. Gottumukkala, D. Notani, O. Rasool, S. Galande, and R. Lahesmaa. 2010. SATB1 dictates expression of multiple genes including IL-5 involved in human T helper cell differentiation. Blood. 116:1443–1453. http://dx.doi.org/10.1182/blood-2009-11-252205

Alvarez, J. D., D. H. Yasui, H. Naid, T. J oh, D. Y. Loh, and T. Kohwi-Shigematsu. 2000. The MAR-binding protein SATB1 orchestrates temporal and spatial expression of multiple genes including IL-5 involved in human T helper cell differentiation. Blood. 116:1443–1453. http://dx.doi.org/10.1182/blood-2009-11-252205

References

Ahlfors, H. A., Linzey, I. L., Elo, S. Tuomela, M. Burute, K. V. Gottumukkala, D. Notani, O. Rasool, S. Galande, and R. Lahesmaa. 2010. SATB1 dictates expression of multiple genes including IL-5 involved in human T helper cell differentiation. Blood. 116:1443–1453. http://dx.doi.org/10.1182/blood-2009-11-252205

Alvarez, J. D., D. H. Yasui, H. Naid, T. J oh, D. Y. Loh, and T. Kohwi-Shigematsu. 2000. The MAR-binding protein SATB1 orchestrates temporal and spatial expression of multiple genes including IL-5 involved in human T helper cell differentiation. Blood. 116:1443–1453. http://dx.doi.org/10.1182/blood-2009-11-252205

Ahlfors, H. A., Linzey, I. L., Elo, S. Tuomela, M. Burute, K. V. Gottumukkala, D. Notani, O. Rasool, S. Galande, and R. Lahesmaa. 2010. SATB1 dictates expression of multiple genes including IL-5 involved in human T helper cell differentiation. Blood. 116:1443–1453. http://dx.doi.org/10.1182/blood-2009-11-252205

Alvarez, J. D., D. H. Yasui, H. Naid, T. J oh, D. Y. Loh, and T. Kohwi-Shigematsu. 2000. The MAR-binding protein SATB1 orchestrates temporal and spatial expression of multiple genes including IL-5 involved in human T helper cell differentiation. Blood. 116:1443–1453. http://dx.doi.org/10.1182/blood-2009-11-252205

Ahlfors, H. A., Linzey, I. L., Elo, S. Tuomela, M. Burute, K. V. Gottumukkala, D. Notani, O. Rasool, S. Galande, and R. Lahesmaa. 2010. SATB1 dictates expression of multiple genes including IL-5 involved in human T helper cell differentiation. Blood. 116:1443–1453. http://dx.doi.org/10.1182/blood-2009-11-252205

Alvarez, J. D., D. H. Yasui, H. Naid, T. J oh, D. Y. Loh, and T. Kohwi-Shigematsu. 2000. The MAR-binding protein SATB1 orchestrates temporal and spatial expression of multiple genes including IL-5 involved in human T helper cell differentiation. Blood. 116:1443–1453. http://dx.doi.org/10.1182/blood-2009-11-252205
expression of multiple genes during T-cell development. *Genes Dev.* 14:521–535.

Balamoots, M.A., N. Tamberg, Y.J. Woo, J. Li, B. Day, T. Kohwi-Shigematsu, and Y. Kohwi. 2012. Satb1 ablation alters temporal expression of immediate early genes and reduces dendritic spine density during postnatal brain development. *Mol. Cell. Biol.* 32:333–347. http://dx.doi.org/10.1128/MCB.05917-11

Bert, A.G., J. Burrows, C.S. Osborne, and P.N. Cockerill. 2000. Generation of an improved luciferase reporter gene plasmid that employs a novel mechanism for high-copy replication. *Plasmid.* 44:173–182. http://dx.doi.org/10.1006/plas.2000.1474

Beyer, M., Y. Thabet, R.U. Müller, T. Sadlon, S. Classen, K. Lahl, S. Basu, X. Zhou, S.L. Bailey-Bucktrout, W. Krebs, et al. 2011. Repression of the genome organizer SATB1 in regulatory T cells is required for suppressive function and inhibition of effector differentiation. *Nat. Immunol.* 12:898–907. http://dx.doi.org/10.1038/ni.2084

Bulger, M., and M. Grouin. 2011. Functional and mechanistic diversity of distal transcription enhancers. *Cell.* 144:327–339. http://dx.doi.org/10.1016/j.cell.2011.01.024

Cai, S., H.J. Han, and T. Kohwi-Shigematsu. 2003. Tissue-specific nuclear architecture and gene expression regulated by SATB1. *Nat. Genet.* 34:42–51. http://dx.doi.org/10.1038/ng1146

Cai, S., C.C. Lee, and T. Kohwi-Shigematsu. 2006. SATB1 packages densely looped, transcriptionally active chromatin for coordinated expression of cytokine genes. *Nat. Genet.* 38:1278–1288. http://dx.doi.org/10.1038/ng1913

Calo, E., and J. Wysocka. 2013. Modification of enhancer chromatin: what, how, and why? *Mol. Cell.* 49:825–837. http://dx.doi.org/10.1016/j.molcel.2013.01.038

Cise, L.I., I. Iredin, S.Z. Cuse, L. Boudarene, A. Senecal, L. Muresan, C. Dugast-Darzacq, B. Hag, M. Dahan, and X. Darzacq. 2013. Real-time dynamics of RNA polymerase II clustering in live human cells. *Science.* 341:664–667. http://dx.doi.org/10.1126/science.1239053

Cook, P.R. 1999. The organization of replication and transcription. *Science.* 284:1790–1795. http://dx.doi.org/10.1126/science.284.5421.1790

de Boer, J., A. Williams, G. Skavdahl, N. Harker, M. Coles, M. Tolani, T. Horton, K. Williams, K. Rodenick, A.J. Potocnik, and D. Kiosonis. 2003. Transgenic mice with hematopoietic and lymphoid specific expression of Cre. *Europ. J. Immunol.* 33:314–325. http://dx.doi.org/10.1002/eji.200330005

Dekker, J., K. Rippe, M. Dekker, and N. Kleckner. 2002. Capturing chromosome conformation. *Science.* 295:1306–1311. http://dx.doi.org/10.1126/science.1067799

Dickson, L.A., T. Jol, Y. Kohwi, and T. Kohwi-Shigematsu. 1992. A tissue-specific MAR/SAR DNA-binding protein with unusual binding site recognition. *Cell.* 70:631–645. http://dx.doi.org/10.1016/0092-8674(92)90432-C

Domen, K., X. Li, and J.L. Weissman. 1998. Systemic overexpression of the TCR beta gene variant sequence on chromosome 7. *Nature Genet.* 19:105–117. http://dx.doi.org/10.1038/ng0798

Domen, J., K.L. Gandy, and I.L. Weissman. 1998. Systemic overexpression of the TCR beta gene variant sequence on chromosome 7. *Nat. Genet.* 19:105–117. http://dx.doi.org/10.1038/ng0798

Jackson, A.M., and M.S. Krangel. 2005. Allele-specific regulation of TCR beta gene variant sequence on chromosome 7. *Nature Genet.* 37:518–519. http://dx.doi.org/10.1038/ng1146

Jackson, A., H.D. Kundin, B. Khor, B.P. Sleckman, and M.S. Krangel. 2005. Regulation of T cell receptor beta allele exclusion at a level beyond accessibility. *Nat. Immunol.* 6:189–197. http://dx.doi.org/10.1038/nii115

Jhungvanwala, S., M.C. van Zeln, M.M. Peak, and C. Murie. 2009. Chromatin architecture and the generation of antigen receptor diversity. *Cell.* 138:435–448. http://dx.doi.org/10.1016/j.cell.2009.07.016

Kim, T.K., M. Hemberg, J.M. Gray, A.M. Costa, D.M. Bear, J. Wu, D.A. Hamrn, M. Laptewicz, K. Barbara-Haley, S. Kuerten, et al. 2010. Widespread transcriptional activity of developmental-regulated enhancers. *Nature.* 465:182–187. http://dx.doi.org/10.1038/nature09033

Koch, F., R. Fenouil, M. Gut, P. Cauchy, T.K. Albert, J. Zacarias-Cabeza, S. Spicuglia, A.L. de la Chapelle, M. Heidemann, C. Hentnarian, et al. 2011. Transcription initiation platforms and GF'T recruitment at tissue-specific enhancers and promoters. *Nat. Struct. Mol. Biol.* 18:956–963. http://dx.doi.org/10.1038/nsmb.2085

Kohwi-Shigematsu, T., K. Poterlowicz, E. Ordinario, H.J. Han, V.A. Bouchkarev, and Y. Kohwi. 2013. Genome-organizing function of SATB1 in tumor progression. *Semin. Cancer Biol.* 23:72–79. http://dx.doi.org/10.1016/j.semcancer.2012.06.089

Krangel, M.S. 2009. Mechanics of T cell receptor gene rearrangement. *Curr. Opin. Immunol.* 21:133–139. http://dx.doi.org/10.1016/j.coi.2009.03.009

Kumar, P.P., P.K. Purby, C.K. Sinha, D. Notani, A. Limaye, R.S. Jayani, and S. Galande. 2006. Phosphorylation of SATB1, a global gene regulator, acts as a molecular switch regulating its transcriptional activity in vivo. *Mol. Cell.* 22:231–243. http://dx.doi.org/10.1016/j.molcel.2006.03.010

Kumar, P.P., O. Bischof, P.K. Purby, D. Notani, H. URLaub, A. Dejean, and S. Galande. 2007. Functional interaction between PML and SATB1 regulates chromatin-loop architecture and transcription of the MHC class I locus. *Nat. Cell Biol.* 9:45–56. http://dx.doi.org/10.1038/ncb1516

Kuo, T.C., and M.S. Schlissel. 2009. Mechanisms controlling expression of the RAG locus during lymphocyte development. *Curr. Opin. Immunol.* 21:173–178. http://dx.doi.org/10.1016/j.coi.2009.03.008

Li, W., D. Notani, Q. Ma, B. Tamaa, E. Nunez, A.Y. Chen, D. Merkurev, J. Zhang, K. Ong, X. Song, et al. 2013. Functional roles of enhancer RNAs for oestrogen-dependent transcriptional activation. *Nature.* 498:516–520. http://dx.doi.org/10.1038/nature09721

Lund, R., H. Ahlfors, E. Kainonen, A.M. Lahesmaa, C. Dixon, and R. Lahesmaa. 2005. Identification of genes involved in the initiation of human Th1 or Th2 cell commitment. *Eur. J. Immunol.* 35:3307–3319. http://dx.doi.org/10.1002/eji.200526079

McMurty, M.T., C. Hernandez-Munain, P. Lauruzsca, and M.S. Krangel. 1997. Enhancer control of local accessibility to V(D)J recombinase. *Mol. Cell.* 17:453–4561

Merklensinger, M., and D.T. Odom. 2013. CTCF and cohesin: linking gene regulatory elements with their targets. *Cell.* 152:1285–1297. http://dx.doi.org/10.1016/j.cell.2013.02.029

Monroe, R.J., F. Chen, R. Ferrini, L. Davidson, and F.W. Alt. 1999. RAG2 is regulated differentially in B and T cells by elements 5' of the...
SATB1 regulates thymocyte \textit{Rag} gene expression | Hao et al.

---

**Promoter**. 
*Proc. Natl. Acad. Sci. USA*. 96:12713–12718. http://dx.doi.org/10.1073/pnas.96.22.12713

**Notani, G., and J.C. Andrau.** 2012. Noncoding transcription at enhancers: general principles and functional models. *Annu. Rev. Genet.* 46:1–19. http://dx.doi.org/10.1146/annurev-genet-110711-155459

**Rosenbloom, K.R., C.A. Sloan, V.S. Malladi, T.R. Dreszer, K. Learned, V.M. Damle, S. Mehta, P.K. Purbe, J. Joseph, and S. Galande.** 2010. Global regulator SATB1 recruits beta-catenin and regulates T(H)2 differentiation in Wnt-independent manner. *PLoS Biol.* 8:e1000296. http://dx.doi.org/10.1371/journal.pbio.1000296

**Yasui, D., M. Miyano, S. Cai, P. Varga-Weisz, and T. Kohwi-Shigematsu.** 2002. SATB1 targets chromatin remodelling to regulate genes over long distances. *Nature*. 419:467–471. http://dx.doi.org/10.1038/nature01312

---

**Bibliography**

**Shih, H.Y., and M.S. Krangel.** 2013. Chromatin architecture, CCCTC-binding factor, and V(D)J recombination: managing long-distance relationships at antigen receptor loci. *J. Immunol.* 190:4915–4921. http://dx.doi.org/10.4049/jimmunol.1300218

---

**Zhang, W., C.L. Sommers, D.N. Burshtyn, C.C. Stebbins, J.B. DeJarnette, R.P. Trible, A. Grinberg, H.C. Tsay, H.M. Jacobs, C.M. Kessler, et al.** 1999. Essential role of LAT in T cell development. *Immunity*. 10:323–332. http://dx.doi.org/10.1016/S1074-7613(00)80032-1