Tissue-specific nuclear architecture and gene expression regulated by SATB1

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Eukaryotic chromosomes are packaged in nuclei by many orders of folding. Little is known about how higher-order chromatin packaging might affect gene expression. SATB1 is a cell-type specific nuclear protein that recruits chromatin-remodeling factors and regulates numerous genes during thymocyte differentiation. Here we show that in thymocyte nuclei, SATB1 has a cage-like 'network' distribution circumscribing heterochromatin and selectively tethers specialized DNA sequences onto its network. This was shown by fluorescence *in situ* hybridization on wild-type and *Satb1*-null thymocytes using *in vivo* SATB1-bound sequences as probes. Many gene loci, including that of *Myc* and a brain-specific gene, are anchored by the SATB1 network at specific genomic sites, and this phenomenon is precisely correlated with proper regulation of distant genes. Histone-modification analyses across a gene-enriched genomic region of 70 kb showed that acetylation of histone H3 at Lys9 and Lys14 peaks at the SATB1-binding site and extends over a region of roughly 10 kb covering genes regulated by SATB1. By contrast, in *Satb1*-null thymocytes, this site is marked by methylation at H3 Lys9. We propose SATB1 as a new type of gene regulator with a novel nuclear architecture, providing sites for tissue-specific organization of DNA sequences and regulating region-specific histone modification.

Introduction

To understand how tissue-specific patterns of gene expression during cellular differentiation are established, much effort has been made towards identifying cell type–specific *trans*-acting DNA-binding factors and *cis*-acting DNA sequences. In addition, recent research has established the biological importance of several enzymes that change chromatin structure by modifying histone tails[1–3] and of chromatin-remodeling enzymes[4–6] that change nucleosome positioning. These are important in establishing an active or inactive state of chromatin, thus regulating gene activity. An important question that has not yet been answered is how genes are found by these factors so that their cell type–specific and stage-specific expression is properly regulated.

One step that may be involved in this process is the assembly of acetyltransferases and different transcription factors at the transcriptional apparatus, resulting in the acetylation of nucleosomes near the binding sites[7,8]. Recent studies on selected gene loci have also shown that eukaryotic chromosomes are organized into discrete domains marked by specific histone modifications to separate independently regulated parts of the genome[9–12]. These studies suggest the importance of higher-order chromatin structure in gene regulation. In addition, emerging evidence suggests that the physical positioning of genes in specific nuclear compartments, such as heterochromatin and the nuclear envelope, affects their activity[13]. All these findings present compelling evidence that several layers of regulatory mechanisms exist to ensure the specific and efficient targeting of transcription factors and chromatin-remodeling and -modifying enzymes to specific regions of genomic DNA.

Gene regulation at the level of chromatin organization in the nucleus is an important part of this multifaceted mechanism and is a growing field of study. There is much to learn as to which proteins are responsible for establishing such nuclear architecture and how chromatin packaging is translated to tissue-specific gene regulation. SATB1 (special AT-rich binding protein 1) was originally identified as a protein that recognizes double-stranded DNA with a high degree of base-unpairing, referred to as base-unpairing regions (BURs; ref. 14). DNA bases of BURs become continuously unpaired when subjected to negative superhelical strain; this property is due to a specialized DNA context (an ATC sequence context) characterized by a cluster of sequence stretches with well mixed As and Ts but either Cs or Gs exclusively on one strand[15]. BURs often contain a core unwinding element, which, when mutagenized, results in the loss of the unwinding property of BURs[15]. This inherent unwinding property has been shown to be important for the augmentation of gene expression in stable transformants[16]. DNA sequences containing BURs, such as those surrounding the immunoglobulin μ heavy-chain enhancer[15,17], are essential for tissue-specific gene expression[18], can extend chromatin accessibility[19] and mediate long-range chromatin modification[19–22]. Some of these functions may be promoted by the interaction between BURs and specific proteins that bind to them[16]. The specific and high-

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affinity binding of SATB1 to the core unwinding elements of BURs requires a BUR-binding domain, a homeodomain and a PDZ domain essential for dimerization of SATB1 (refs. 23–25). SATB1 is expressed in a lineage-specific manner, primarily in the T-cell lineage and in certain other tissue-specific progenitor or precursor cells[14,26,27]. Although several BUR-recognizing proteins have been identified to date[28–30], BRIGHT is the only other one that is tissue-specific, and it is expressed in B cells[31].

Satb1-knockout mice live to 3 weeks after birth. Their T-cell development is blocked mainly at the $CD4^+CD8^+$ double-positive stage, and the few peripheral T cells found in these mice undergo apoptosis on mitogen stimulation[27]. *Satb1* ablation by gene targeting led to gene dysregulation affecting hundreds of genes (at least 2% of total genes, either expressed or silent), including genes important for T-cell function, such as interleukin receptor 2a (*Il2ra*) and *Il7r*[27]. In *Satb1*-null thymocytes, expression of many genes is temporally or spatially incorrect.

By fluorescence *in situ* hybridization (FISH) analysis, SATB1-bound genomic sequences (SBSs) *in vivo* isolated from Jurkat lymphoblastic cells were located at the bases of DNA 'halos' generated by salt extraction of interphase nuclei, and these signals were retained even after extensive digestion with DNase 1 (ref. 32). DNA halos represent loops of genomic DNA emanating from the residual nuclei, anchored at their bases to the components of nuclei that are resistant to salt extraction and digestion with DNase 1. These data show that SATB1 is a cell type–specific protein that binds to the bases of chromatin loops.

Most recently, SATB1 has been found to target multiple chromatin-remodeling complexes to specific genomic sites to regulate chromatin structure and expression of genes over long distances. In the *Il2ra* locus, SATB1 recruits the histone deacetylase contained in the NURD chromatin-remodeling complex to an SBS and mediates the specific deacetylation of histones in a large domain in the locus. SATB1 also targets ACF1 and ISWI, subunits of CHRAC and ACF nucleosome-mobilizing complexes, to the specific site in the *Il2ra* locus and regulates nucleosome positioning over 7 kb. This activity is precisely linked to proper regulation of *Il2ra* during thymocyte differentiation[33].

Here we show that SATB1 is a new type of gene regulator that functions in tissue-specific organization of DNA sequences. SATB1 has an unusual cage-like distribution in thymocyte nuclei and resists salt extraction. SATB1 is necessary for folding chromatin by tethering specialized DNA sequences (ATC sequences) onto its network; this anchoring of DNA by SATB1 is tightly correlated with proper regulation of genes that are located as far as 50 kb distal to SBSs. Furthermore, SATB1 binding to a single SBS establishes specific histone modifications over a region containing genes whose expression is SATB1-dependent. Our data suggest a novel mechanism of tissue-specific gene regulation in which a single protein can establish a unique intranuclear architecture by providing docking sites for specialized DNA sequences and enzymes that modify chromatin activity.

Results

SATB1 has a cage-like network distribution in thymocyte nuclei

To study the role of SATB1, we first examined its nuclear distribution pattern by immunostaining of thymocytes with a rabbit polyclonal antibody against SATB1 (1583). SATB1 had a dense three-dimensional cage-like or chicken-wire-like distribution embracing dense chromatin regions that were stained heavily with DAPI (Fig. 1). These dense chromatin regions were also stained with an antibody for heterochromatin-associated M31 protein (Fig. 1*d–f*). We refer to

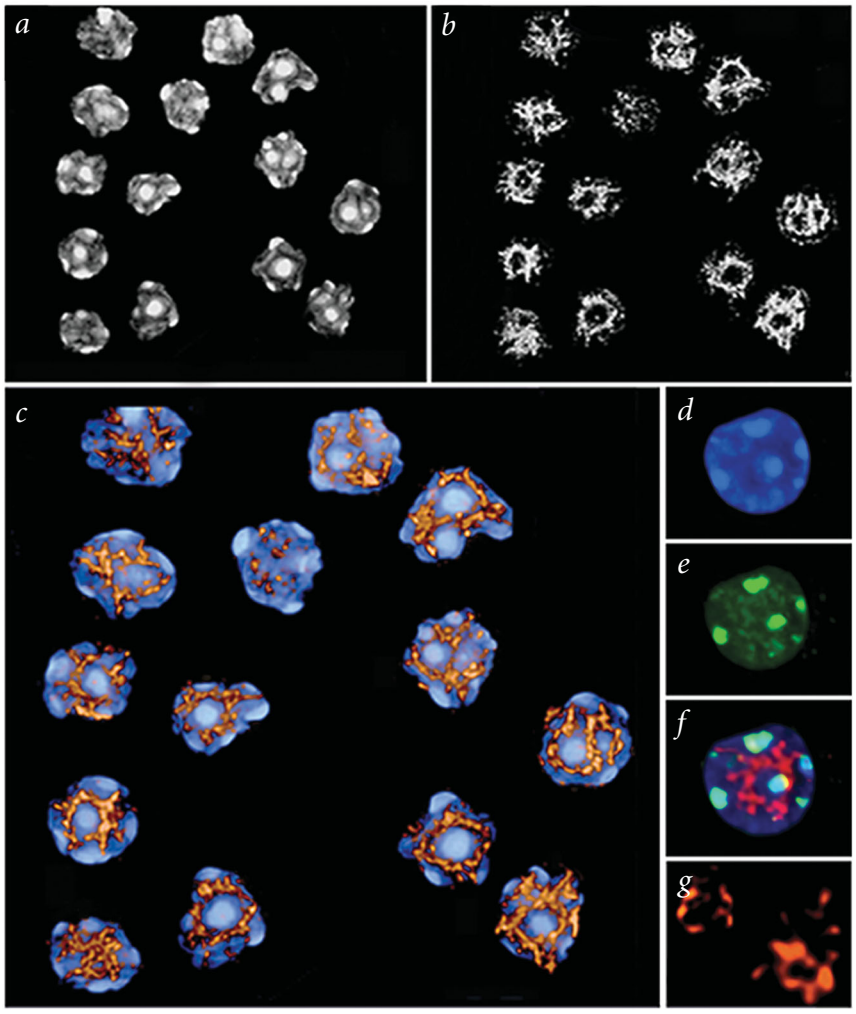


Fig. 1 Identification of a cage-like network structure of SATB1 in thymocytes. Thymocytes isolated from a 2-wk-old mouse were immunohistochemically labeled with antibody against SATB1 (antibody 1583; ref. 14) and DAPI. The images were collected by DeltaVision microscope. Black and white image of cells stained with DAPI (***a***), cells stained with antibody against SATB1 (***b***) and the merged image in pseudo-color, DNA in light blue and SATB1 in gold-orange (***c***), are shown. SATB1 forms a three-dimensional network that has the shape of a cage surrounding dense regions of chromatin. A thymocyte cell stained with DAPI (blue; ***d***), the same cell stained with antibody against M31 (green; ***e***) and the merged image of the cell stained with DAPI and antibodies against M31 and against SATB1 (red; ***f***) are shown. Thymocyte nuclei, which were salt-extracted and digested with DNase 1, were similarly stained (***g***). No staining by DAPI was detected after digestion with DNase 1. Although some diffusion of SATB1 signals was evident, the basic structure remained intact. Two representative residual nuclei samples are shown.

this particular distribution as a SATB1 'network'. In thymocytes, SATB1 is an abundant protein and forms at least a dimer, possibly multimers, through its PDZ-like domain[25]. After salt extraction and extensive digestion with DNase 1, a substantial amount of SATB1 and the SATB1 network persisted, although SATB1 seemed somewhat diffuse (Fig. 1*g*). Because this unique network of SATB1 is mostly resistant to salt extraction and digestion with DNase 1, and because our previous FISH experiments showed that *in vivo* SBSs also remained strongly anchored to residual nuclei that are similarly treated[32], chromatin fibers are probably anchored at these sequences to the SATB1 network circumscribing heterochromatin. To show unambiguously that SATB1, but not other proteins, is necessary for folding chromatin by making its target sequences chromatin-loop bases, we compared wild-type and *Satb1*-null thymocytes as described below.

SATB1 folds chromatin at an SBS 5′ of *Myc*

To examine whether the SATB1 network has an active role in chromatin folding and gene regulation, we first focused on the *Myc* gene, which is dysregulated in *Satb1*-null thymocytes[27]. We searched for a site in the human *MYC* locus to which SATB1 specifically binds *in vivo*. Because the sequences to which SATB1 binds have the potential to become continuously base-unpaired under negative superhelical strain[14,15], potential SBSs can be detected by reacting supercoiled DNA containing the sequences with chloroacetaldehyde, which reacts specifically with unpaired DNA[34]. We identified an ATC sequence stretch of 24 bp located at –1,531 to –1,507 from the transcription start site of *MYC* that was uniquely reactive with chloroacetaldehyde (data not shown). A similar ATC sequence stretch was found at the orthologous location in the mouse *Myc* gene.

To examine whether this sequence is bound by SATB1 in mouse thymocytes, we carried out a special chromatin immunoprecipitation (ChIP) assay, which involves formaldehyde-crosslinking of cells followed by centrifugation through a 5–8 M urea gradient to purify crosslinked chromatin. This method is particularly suited for determining *in vivo* target sequences of DNA-binding proteins[35]. With a specific set of primers, we verified that the 260-bp sequence containing the 24-bp ATC sequence stretch was immunoprecipitated specifically by the antibody against SATB1 from a pool of chromatin fragments formaldehyde-crosslinked in thymocytes (Fig. 2*a*). SBS-336 (listed in Fig. 4*a*) was also present in this pool of chromatin fragments, whereas a *Zap70* coding sequence, which does not have any SBSs, was absent. In the chromatin fragments immunoprecipitated using the control preimmune serum of the same rabbit that generated the antibody against SATB1, the 24-bp *Myc* upstream sequence was absent. These data indicate that the upstream ATC sequence stretch is bound to SATB1 *in vivo* in thymocytes.

We carried out amplified FISH using as a probe a *Myc* upstream DNA fragment of 700 bp containing the 24-bp ATC sequence as the sole SBS from the chloroacetaldehyde analysis described above. We carried out tyramide-amplified FISH with slide preparations of nuclei, of salt-extracted nuclei generating

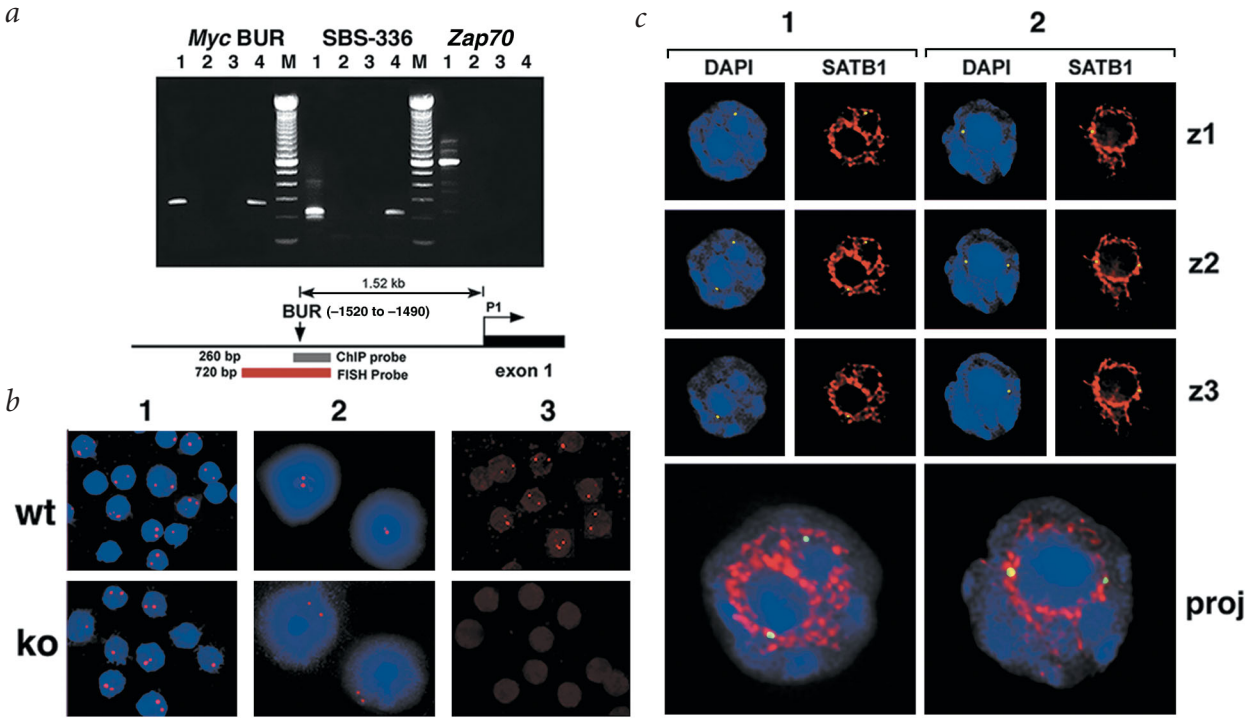


Fig. 2 SATB1 actively anchors the *Myc* upstream sequence onto its network. ***a***, ChIP was done with either preimmune serum or antibody against SATB1 (crude and purified) on formaldehyde-crosslinked thymocytes to determine *in vivo* binding of a 260-bp sequence containing a BUR, which is located 1.5 kb 5′ of *Myc*. Specific oligomers derived from SBS-336 isolated as *in vivo* SBS (see Fig. 4) was used as a positive control, and the *Zap70* coding sequence was used as negative control. PCR amplification using specific set of primers was done with genomic DNA control (lane 1), water control (lane 2), chromatin fraction immunoprecipitated with preimmune serum (lane 3) and chromatin fraction immunoprecipitated with antibody against SATB1 (lane 4). M, molecular weight marker. A map indicating the BUR site and the probes used are shown. ***b***, Tyramide-amplified FISH was done with a 700-bp probe containing the SATB1-binding sequence identified 5′ of *Myc*. Thymocyte nuclei (1) isolated from either wild-type (wt) or *Satb1*-knockout (ko) thymus were spun down onto slides and extracted *in situ* with 2 M NaCl to produce DNA halos (2) and then further digested with DNase 1 (3). DNA is stained with DAPI (blue), and hybridization signals are visualized by rhodamine-conjugated ExtrAvidin (red). ***c***, A combination of immunostaining with antibody against SATB1 and tyramide-amplified FISH using the 700-bp *Myc* upstream sequence as probe was done on wild-type thymocytes. DNA is stained with DAPI, hybridization signals were visualized by ExtrAvidin conjugated with fluorescein isothiocyanate (green) and immunostaining was visualized by Alexa-594–conjugated goat antibody against rabbit IgG (red). Different optical sections (z1, z2 and z3) separated by 0.2-μm spacing for the two cells (1) and (2) are shown. The large images below (proj) are the composite of these optical sections.

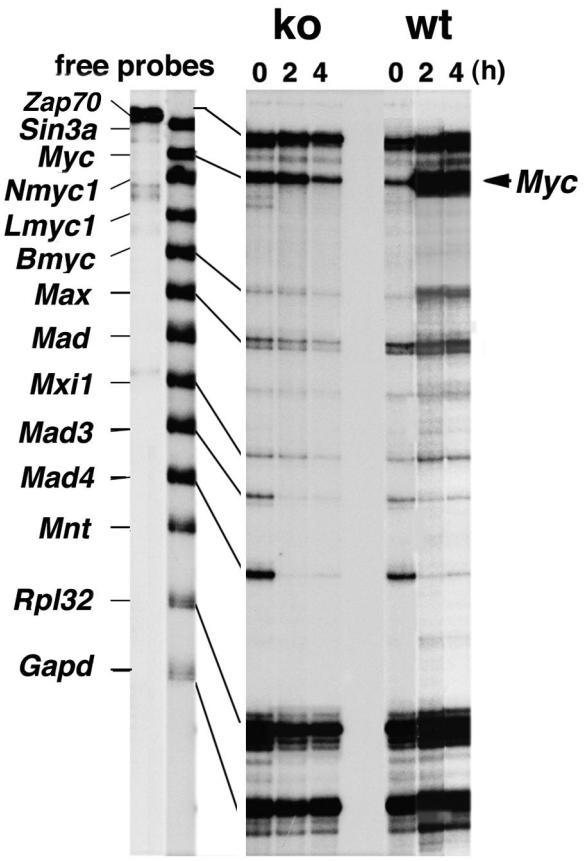


Fig. 3 SATB1 regulates *Myc* in thymocytes. RNase protection assay was done using the *Myc* multi-probe template to determine the transcript levels of *Myc* 0 h, 2 h and 4 h after stimulation with PMA and ionomycin from total RNA isolated from either wild-type (wt) or *Satb1*-knockout (ko) thymocytes. *Zap70* transcript was monitored as a loading control[27].

DNA halos surrounding residual nuclei and of protein-extracted and DNase 1–digested nuclei (Fig. 2*b*) as described[36]. We detected similar FISH signals (one for each allele) in both wild-type and *Satb1*-null nuclei preparations (Fig. 2*b*). We prepared DNA halos by extracting nuclei cytospun onto slides with 2 M NaCl *in situ* to remove histones and most of the non-histone proteins from nuclei, allowing DNA loops to flow out from the residual nuclei. In halo preparations from wild-type nuclei, the FISH signals were retained in the residual portion of the nuclei. In contrast, in halo preparations from *Satb1*-null nuclei, the FISH signals were found mostly in the distended DNA loop (halo) regions (Fig. 2*b*). After extensive digestion with DNase 1 to remove most DNA from the nuclei, the FISH signals were detected in the wild-type nuclei, where the SATB1 network persisted. In the absence of SATB1, however, the FISH signals were completely lost (Fig. 2*b*). These data show that the *Myc* SBS locus is no longer at the base of chromatin loops after ablation of SATB1, indicating that SATB1 is necessary to make this site a chromatin-loop base. Our data eliminate the possibility that SATB1 binds the *Myc* upstream site that had been already anchored by other protein(s) in the nucleus resistant to salt extraction. To show directly that the *Myc* upstream site was indeed anchored onto the SATB1 network, we immunostained wild-type thymocytes with antibody against SATB1 and then carried out the FISH experiment and examined the images at different optical sections by DeltaVision microscope. The FISH signals colocalized with the SATB1 network in all thymocytes that we examined. Two representative cells are shown (Fig. 2*c*). These data indicate that the SATB1 network contributes to higher-order packaging of chromatin by tethering specific genomic sites to itself.

SATB1 regulates induction of *Myc* in thymocytes

Our data showed that SATB1 regulates the three-dimensional organization of chromatin at the *Myc* locus. Therefore, we further studied *Myc* gene regulation by SATB1. *Myc* is normally expressed at low levels in wild-type thymocytes, most of which are resting $CD4^+CD8^+$ thymocytes, whose mRNA level is highly induced after mitogenic stimulation, such as with the calcium ionophore ionomycin plus 12-phorbol 13-myristate acetate (PMA; ref. 37). In *Satb1*-null thymocytes, however, *Myc* is already expressed at moderately high levels (about 4 times higher than in wild-type) before stimulation[27]. We carried out RNase protection assays before and after thymocytes were stimulated with PMA and ionomycin. Two hours after stimulation, the transcript level in wild-type thymocytes was more than 10 times higher, and this induction persisted to 4 hours after stimulation. In contrast, *Myc* was not induced in *Satb1*-null thymocytes, and its transcript levels rapidly decreased during the time course of stimulation (Fig. 3). These results show that in the absence of SATB1, *Myc* is dysregulated and uninducible after mitogenic stimulation. Few of the pathways regulating Myc expression have been fully elucidated, but many growth-factor and cytokine-signaling pathways seem to converge to regulate the Myc protein level, which in turn influences cell growth and proliferation by direct activation of genes involved in these processes[38]. Our data show that SATB1 is necessary for *Myc* induction in thymocytes in response to mitogenic stimuli. Because Myc is important for thymocyte maturation[39], dysregulated *Myc* expression may, at least in part, explain the impaired T-cell development in *Satb1*-null mice.

In vivo SBSs become chromatin-loop bases by active tethering to the SATB1 network

Having found that anchoring the specific *Myc* upstream sequence onto the SATB1 network is associated with the inducibility of *Myc*, we wished to verify that such anchoring could be a common mechanism by which SATB1 regulates genes. We tested this by isolating individual *in vivo* SBSs from thymocyte nuclei and analyzing genes located within 100 kb of each SBS. From such experiments, we expected to learn where in the genome relative to the target gene(s) the chromatin fiber is anchored to the SATB1 network and what the consequence of this might be for transcription of each target gene. We cloned and identified a series of genomic loci that are bound to SATB1 *in vivo* in mouse thymocytes. This was achieved by ligation-mediated PCR amplification (LM–PCR) of crosslinked chromatin fragments generated from thymocytes that were purified by centrifugation through a urea gradient followed by digestion with a restriction enzyme and immunoprecipitation with antiserum against SATB1 (ref. 35). Conditions were optimized so that no LM–PCR products could be seen from the pre-immune serum immunoprecipitates. No two SBSs cloned were identical, as they are represented by a specialized DNA context rather than a primary sequence consensus. Each cloned DNA has a high affinity for SATB1 and is characterized by the ATC sequence context (Fig. 4*a*).

Among a series of *in vivo* SBSs cloned, we randomly chose six DNA fragments (SBS-418, SBS-336, SBS-439, SBS-T4, SBS-372 and SBS-343) as FISH probes. Similar to the case for the *Myc* locus, in wild-type thymocytes, all six DNA fragments were found at the bases of chromatin loops in the halo preparation and were retained in the salt-extracted and DNase 1–digested nuclei. In contrast, in *Satb1*-null thymocytes, these fragments were detached from the bases and found in the halo portion and were lost after digestion with DNase 1. Representative FISH data for wild-type versus *Satb1*-

knockout halos are shown for SBS-418 and SBS-T4 (Fig. 4*b*). At least 200 halos were scored and most (>80%) had this pattern; this was identical for all six SBSs tested. After further digestion with DNase 1, the hybridization signals persisted only in wild-type thymocyte nuclei (data not shown). These data, together with the result from the SBS 5′ of *Myc*, indicate that in wild-type thymocytes all *in vivo* SBSs examined are anchored to the SATB1 network at the bases of chromatin loops.

a

SBS	length (kb)	k_d (nM)	sequence feature
T4	0.5	4-8	ATC/p53 intron 1-like
1	0.4	4-8	ATC
37	1.0	1	ATC
284	1.2	2-4	ATC/homopurine
336	1.1	1	ATC/homopurine
343	1.5	2-4	ATC
372	1.0	4-8	ATC
376	0.8	4	ATC/(CA)n, (AT)n
418	1.2	2	ATC/(GA)n
439	1.0	0.5	ATC/telomere flank
461	1.0	4	ATC/L1

b

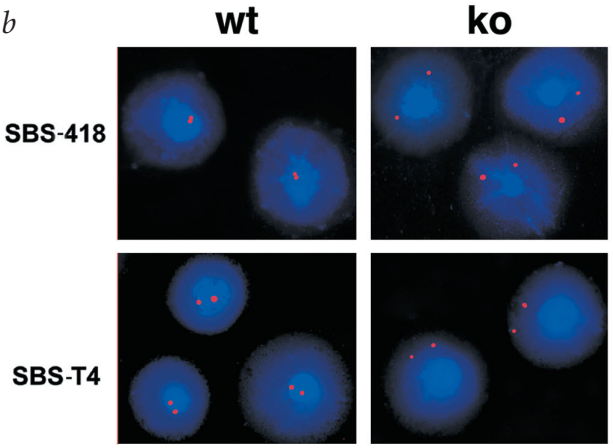


Fig. 4 Isolation and characterization of individual genomic sequences that bind to SATB1 *in vivo* in mouse thymocytes. ***a***, Individual sequences were cloned from an *in vivo* crosslinked chromatin fraction specifically immunoprecipitated by antibody against SATB1 according to a published method[35]. Dissociation constant (k_d) values were determined as described[32] and the sequence characteristics are shown. All sequences have the ATC sequence context. ***b***, Amplified FISH data is shown using SBS-418 and SBS-T4 as probes for *in situ* prepared DNA halos from wild-type (wt) and *Satb1*-knockout (ko) thymocytes as described for Figure 2. Similar analyses were done for the four additional SBSs, and identical results were obtained (data not shown).

SATB1-dependent expression of genes within 100 kb of SBSs

If specific folding of chromatin at SBSs to the SATB1 network is important for the proper regulation of genes, those genes found in close proximity (within 100 kb) of each SBS in *Satb1*-null thymocytes are expected to be dysregulated, similar to *Myc*. To test this idea, we obtained BAC and P1 clones containing roughly 50–165 kb of genomic inserts with each of the SBSs. We sequenced two BAC clones, BAC-418 (165 kb) and BAC-439 (164.9 kb), and one P1 clone, P1-T4 (53.8 kb). BLAST analysis of BAC-418 showed that it contains the mouse gene BC030338, homologous to the human *KIAA1598* gene, which is predominantly expressed in the brain. Using 418-22, which contains two exons (Fig. 5*a*), as a probe, we carried out an RNase protection assay and confirmed that in wild-type mice, this gene was expressed in brain but not in thymus (Fig. 5*b*). Although there was no difference in the transcription level of this gene in brain tissue of wild-type versus *Satb1*-null mice, this gene was ectopically expressed in thymus of *Satb1*-null mice (Fig. 5*b*). This means that SATB1 binding to SBS-418 affects a gene located 57.6 kb from the binding site.

In addition to the mouse homolog of *KIAA1598*, we found another gene, the mouse homolog of *EEF1G* (encoding translation elongation factor γ), within the 165-kb genomic insert of the BAC-418 clone, located 46 kb distal to SBS-418 (ref. 40). EEF1G was expressed at similar levels in the brains of wild-type and *Satb1*-null mice but at a moderately higher level in *Satb1*-null thymocytes than in wild-type thymocytes (Fig. 5*b*). Our data strongly suggest that SATB1 binding to SBS-418 is essential in the regulation of two genes located in a large domain around this binding site, regardless of the direction of the transcripts (Fig. 5*a*).

We found that genes in other SATB1-bound loci were also dysregulated. In P1-T4, there is a UniGene cluster (GenBank sequences that are experimentally determined to represent a unique gene; Mm.24739) and two other genes (BC018474 and AF250844) on one side of SBS-T4 and an expressed-sequence tag (EST; AW209596) on the other side (Fig. 5*c*). RNase protection assays showed that the transcript levels of the three genes in the UniGene cluster were lower by various degrees in *Satb1*-null thymocytes compared with wild-type control. By contrast, expression levels of the two other genes more distal to SBS-T4 and the EST located on the other side of SBS-T4 were independent of SATB1 expression. This is probably not because these genes are positioned farther away from SBS-T4 than is the UniGene cluster, as SATB1 can regulate genes roughly 40–50 kb distal to its binding site, as shown for SBS-418. Among the genes in the UniGene cluster, the most distal gene (AU051681) to SBS-T4 was most downregulated after ablation of SATB1 in thymocytes (Fig. 5*d*). The transcript levels of all three genes were unaltered in brain (Fig. 5*d*) and liver (data not shown). In sharp contrast to the SBS-418 locus where SATB1 has a repressive effect, SATB1 acts as a transcriptional activator at the SBS-T4 locus. Therefore, SATB1 is not always a repressor as determined by other assay systems[41,42]. That SATB1 binding to SBS-T4 influences a discrete set of genes and that the effect is not propagated to other surrounding genes may be due to other forms of protein–DNA interaction, such as those mediated by CTCF, which is known to block enhancer action[43]. The results obtained from the analysis of BAC-418 and P1-T4 clones show that genes that are dysregulated by SATB1 ablation can be found within 4–60 kb of SBSs, and the entire loci of these genes can extend more than 100 kb from an SBS. As a consequence of anchoring to the SATB1 network in thymocytes, some associated genes are properly repressed, whereas others are activated or become inducible on stimulation, such as *Myc*.

SATB1 binding to SBS-T4 mediates the formation of a site-specific histone code

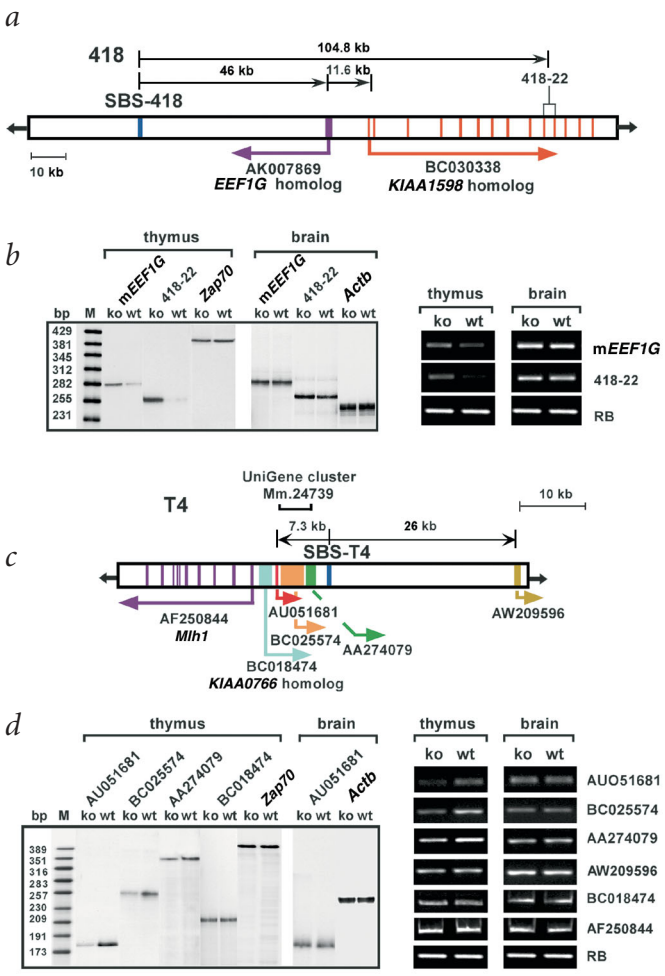
At the SBS-T4 locus, the SATB1-dependent effect on expression is restricted to a UniGene cluster (Mm.24739) located within 4–7.3 kb of SBS-T4. Expression of genes in this cluster was affected after ablation of SATB1 to varying levels, but expression of other genes immediately outside this cluster was unaffected. This selectivity suggests the existence of a functional chromatin domain independent of the neighboring region to regulate this particular set of genes. Such domains may be marked by specific histone modifications. Various types of histone modifications, especially acetylation and methylation, have been correlated with the transcriptional status of chromatin domains. Numerous studies have established that in the neighborhood of transcriptionally active promoters and enhancers, lysine residues on the N-terminal tails of histones H3 and H4 are more highly acetylated than those in transcriptionally inactive regions[2]. We tested

whether SATB1 binding to a specific genomic site *in vivo* could influence the local histone modifications associated with correct expression of the gene(s) in the locus.

We studied the histone modification status over a 70-kb region in the P1-T4 genomic insert encompassing two genes (BC018474 and AF250844) distal to SBS-T4, the UniGene cluster (Mm.24739), SBS-T4 and the EST (AW209596). We first determined the levels of acetylation of histone H3 across this region by ChIP assay using antibodies against histone H3 diacetylated at Lys9 and Lys14. We used a high-resolution, semi-quantitative PCR to analyze immunoprecipitated chromatin fractions using primers to amplify fragments of 400–600 bp (some overlapped) from this 70-kb region. In wild-type thymocytes, there was a peak of acetylation at Lys9 and Lys14 of histone H3 over SBS-T4 (primer sets 21 and 22 overlap with SBS-T4; Fig. 6*a*). The 4.3-kb region (primer sets 20–24), including the SBS-T4 site, had a marked difference in the level of acetylation of chromatin between wild-type and *Satb1*-null thymocytes. Adjacent to this narrowly defined region, an additional smaller peak of hyperacetylation was detected coinciding with the gene (AU051681) in the UniGene cluster that was most downregulated after ablation of SATB1. The regions containing genes (AF250844, BC018474, AW209596) outside the UniGene cluster were hypoacetylated. In *Satb1*-null thymocytes, histone acetylation was low throughout this 70-kb region. Thus, two peaks of high acetylation of histone H3 in a small, well defined region of approximately 10 kb in wild-type thymocytes were greatly diminished in *Satb1*-null thymocytes. Our data show that SATB1 binding to SBS-T4 has a strong impact on the histone acetylation status of chromatin, making this particular site and the adjacent region uniquely hyperacetylated. This phenomenon is precisely correlated with gene expression dependent on SATB1.

In addition to histone acetylation, recent studies have linked histone methylation to the regulation of gene expression[3]. Methylation of histone H3 at Lys4 and Lys9 is associated with transcriptionally active and silent chromatin, respectively[9,12]. We used ChIP assays to determine patterns of histone methylation at Lys4 within the same 70-kb region analyzed for histone H3 acetylation. For wild-type thymocyte chromatin, we observed a strong peak of histone methylation at Lys4 over SBS-T4, similar to that observed for histone H3 acetylation at Lys9 and Lys14 (Fig. 6*b*). These data are consistent with data from the chicken β-globin locus where the patterns of H3 acetylation at Lys9 and Lys14 coincided with H3 methylation at Lys4 (ref. 12).

We observed an unexpected switch in histone modification between wild-type and *Satb1*-null chromatin when we extended the ChIP analysis to examine H3 methylation at Lys9 over the same locus. In contrast to wild-type thymocytes, in which we detected no local enrichment of Lys9 methylation, we detected a peak of histone H3 methylation at Lys9 over SBS-T4 in *Satb1*-null thymocytes, further extending into the UniGene cluster (Fig. 6*c*). These results indicate that SATB1 binding to SBS-T4 is necessary for preventing methylation at Lys9 as well as facilitating its acetylation. Our data show that the binding of SATB1 to a single genomic site, SBS-T4, mediates the formation of a specific chromatin structure marked by a particular histone code over a restricted region containing genes whose expression is SATB1-dependent.



Discussion

There is growing evidence that the transcriptional activity of genes might be influenced by nuclear organization[13]. Genes positioned near heterochromatin are often transcriptionally inactive, and a lymphoid cell lineage–specific protein, Ikaros, is associated with heterochromatin foci, colocalizing with transcriptionally inactive genes[44]. For the human β-globin locus, its localization away from centromeric heterochromatin is required to achieve a nuclease-sensitive, open chro-

Fig. 5 SATB1 regulates expression of distant genes. ***a***, Schematic diagram of the gene locus on the BAC-418 clone. In a total of 165,033 nucleotides sequenced, two UniGene clusters, Mm.42960 and Mm.37338, were found near SBS-418. The mouse gene (AK007869) in the cluster Mm.42960 is a mouse homolog of *EEF1G*. The mouse gene (BC030338) in UniGene Mm.37338 is a mouse homolog of *KIAA1598*. ***b***, Transcription analysis for mouse homologs of *KIAA1598* and *EEF1G*. RNase protection assay and semiquantitative RT–PCR were done using RNA isolated from thymus and brain from 2-wk-old *Satb1*-knockout (ko) and wild-type (wt) mice. The results of the RNase protection assay for the *KIAA1598* homolog monitored by the probe 418-22 and the *EEF1G* homolog (m*EEF1G*) are shown on the left panel. The RT–PCR results for these genes are shown on the right panel. Expression of *Zap70* and *Actb* were used as loading controls for RPA, and expression of retinoblastoma gene (RB) was used as a loading control in RT–PCR because expression of these genes are unaltered in *Satb1*-knockout thymocytes[27]. M, molecular weight marker. ***c***, Schematic diagram of the gene locus on the P1-T4 clone. The sequence of 53,760 nucleotides identified a UniGene cluster (Mm.24739) containing at least three independent genes near SBS-T4 (AU051681, BC025574 and AA274079) and three other genes on both sides of SBS-T4 (*Mlh1* and *KIAA0766* homolog (BC018474) and an EST (AW209596)). ***d***, Transcription analysis of genes near SBS-T4. Representative RNase protection results for genes AU051681, BC025574 and AA274079. BC018472 in the thymus and the AU051681 in brain are shown on the left panel. The RT–PCR results for all genes indicated in the map (*c*) in thymus and brain are shown on the right panel. The loading controls used are the same as in *b*. M, molecular weight marker.

matin configuration, which is marked by acetylation of histones H3 and H4 (ref. 10). In *Drosophila melanogaster*, the gypsy insulator sites are brought together by proteins interacting with them and are attached to a fixed perinuclear substrate[45]. Here we describe an unprecedented intranuclear localization of a tissue-specific protein, SATB1, tethering specialized DNA sequences to its cage-like network and controlling histone modifications to ensure accurate expression of its target genes. Our data present a novel paradigm in which a single protein can regulate tissue-specific gene expression through the active organization of higher-order chromatin structure.

The unique cage-like distribution pattern of SATB1 circumscribing heterochromatin regions, which is not easily breakable by various biochemical treatments, strongly suggests that SATB1 is both an important determinant and a primary component of a subnuclear structure. To confirm the function of SATB1 in chromatin organization and its effect on gene expression, we pursued two complementary approaches. One entailed starting with a gene known to be dysregulated in *Satb1*-knockout thymocytes, such as *Myc*, and identifying an SBS associated with the locus. The other approach involved blindly cloning a series of SBSs from the total thymocyte genomic DNA *in vivo*, identifying genes within 100 kb of each SBS and determining whether their expression was altered in *Satb1*-knockout thymocytes. All SBSs identified by either approach contained the ATC sequence context and localized to the bases of chromatin loops by tethering to the SATB1 network. In the absence of SATB1, these sites were released to the loop regions, indicating that the SATB1 network provides docking sites for chromosomal fibers by actively tethering specific genomic sequences and contributes to higher-order packaging of chromatin.

We show that the attachment of an SBS located 1.5 kb upstream of the *Myc* promoter to the SATB1 network is essential for induction of *Myc* transcription in response to mitogen stimulation. Notably, far upstream sequence element–binding protein, FBP, which regulates *Myc* activity, binds to this same sequence when it is single-stranded[46]. This is in sharp contrast to SATB1, which binds double-stranded BURs, presumably recognizing the altered sugar-phosphate backbone structure of the ATC sequence stretches[14]. Owing to this apparent difference in the conformation specificity, FBP and SATB1 probably do not occupy the same BUR site simultaneously. Our data show that the basal level of *Myc* transcription is considerably higher in *Satb1*-null thymocytes, indicating that SATB1 is not necessary for active *Myc* transcription *per se*. On the other hand, FBP is essential for active transcription of *Myc*[47]. We speculate that SATB1 and the transcription factor FBP act at different levels. SATB1 may establish chromatin structure suited for proper regulation of this locus, including its inducibility. FBP may act as an important transcription factor supporting active *Myc* expression on such chromatin poised for transcription. Because BUR sequences are highly potentiated for base unpairing, such sequences may adopt single- or double-stranded structures dynamically depending on local negative supercoiling, which in turn depends on active transcription of a downstream gene[48]. As with the BUR located 5′ of *Myc*, BURs with high flexibility in DNA conformation may serve as preferred sites for assembling multiple DNA-binding regulatory factors.

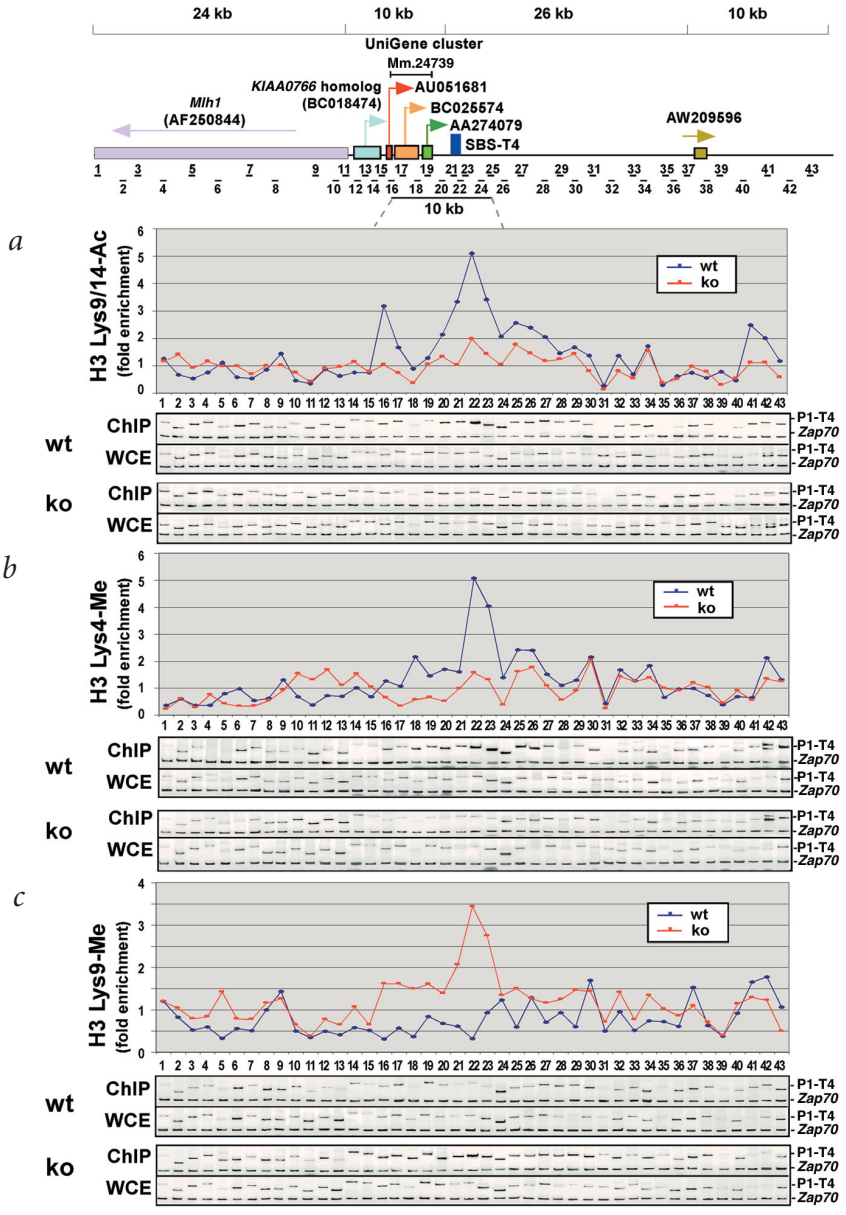


Fig. 6 SATB1 creates a chromatin domain marked by specific histone modification. DNA isolated from immunoprecipitated chromatin (ChIP) or whole-cell crude extracts (WCE) was subjected to multiplex PCR to amplify DNA fragments from the SBS-T4 locus. ChIP was done using antibody against H3 acetylation (Ac) at Lys9 and Lys14 (*a*), antibody against H3 methylation at Lys4 (*b*) and antibody against H3 methylation (Me) at Lys9 (*c*). PCR amplification was done using oligonucleotide primer sets indicated by numbers 1 through 43 shown below the map on the SBS-T4 locus. Red line on graph indicates histone modification status at each site of *Satb1*-null chromatin, and blue line indicates that of wild-type chromatin.

We identified a series of putative target genes for SATB1 by sequencing a genomic region of roughly 100–160 kb containing individually cloned SBSs. These include a neuron-specific gene associated with SBS-418, which we found to be properly repressed in wild-type thymocytes but ectopically transcribed in *Satb1*-null thymocytes. This is similar to the previously analyzed T cell–specific gene *Il2ra*, for which SATB1 acts as a repressor in $CD4^+CD8^+$ thymocytes[33]. Previous work showed also that SATB1 acts as a repressor[41,42]. The role of SATB1 on *Myc*, however, showed that SATB1 is not always a repressor. In fact, we identified SBS-associated genes that are actively transcribed in the presence of SATB1 and downregulated after ablation of SATB1, indicating that SATB1 can also function as a transcriptional activator in some situations.

On the basis of the finding that SATB1 targets chromatin-remodeling and -modifying factors, we propose that SATB1 mainly functions as a landing platform to assemble factors, recruiting either repressor or activator complexes depending on specific genomic sites or need. The fact that SATB1 can act as either a transcriptional activator or a repressor naturally raises the question as to how it chooses its function for any given gene. Other regulatory mechanisms that control the specific transcriptional outcome probably exist upstream of SATB1. One possibility might be that, at any given site, SATB1 might interact specifically with other factors binding to the neighboring sequences. Alternatively, SATB1 might itself be differentially modified at specific sites, resulting in different specificities for recruiting transcriptional regulators. This question will be explored in future work.

Among several genes identified surrounding SBS-T4, only genes in the UniGene cluster were dependent on SATB1. After ablation of SATB1, these genes were downregulated. Emerging evidence strongly suggests that distinct H3 and H4 tail modifications specify a histone code that dictates the regulatory features of a gene[2]. Using the ChIP assay across the genomic locus of roughly 70 kb containing SBS-T4, we show that the histone modification pattern in a 10-kb region containing SBS-T4 and its neighboring UniGene cluster is strictly dependent on the presence or absence of SATB1. When SATB1 binds to SBS-T4, this region is marked by high acetylation at H3 Lys9 and Lys14, as well as methylation at Lys4 of histone H3, with peaks at SBS-T4 and the neighboring sequences. By contrast, when SBS-T4 is not bound to SATB1, as in *Satb1*-null thymocytes, SBS-T4 is highly methylated at Lys9 of histone H3. Therefore, SATB1 binding to a single locus, SBS-T4, establishes a distinct chromatin region and regulates genes in that region.

It has been reported that the chromodomain-containing transcriptional repressor HP1 binds selectively to histone H3 methylated at Lys9 and associates with histone methyltransferases (SUB39 enzyme; refs. 2,3). Our data predict that in the absence of SATB1, the SBS-T4 site is bound to some methyltransferase so that histone H3 is methylated at Lys9 and subsequently bound to HP1. The SBS-T4 site is probably poised for the spread of heterochromatinization. When SATB1 binds to SBS-T4, however, there is a marked switch from methylation to acetylation at Lys9 of histone H3, and the methylation site of histone H3 switches from Lys9 to Lys4. Thus, SATB1 binding to SBS-T4 leads to an open chromatin domain, and the histone modification patterns precisely correlate with the transcriptional status of the genes in this region.

Changes in local histone modification resulting from a single protein binding to a genomic site is reminiscent of a recent study of the retinoblastoma co-repressor protein[49]. Retinoblastoma recruits SUV39/HP1 to the *Ccne* gene (encoding cyclin E), methylating histone H3 at Lys9. Subsequent association of the HP1 with the *Ccne* promoter represses this gene. In the case of the SBS-418 locus, where a neuron-specific gene is derepressed in *Satb1*-null thymus, the histone modification pattern of at least the 5-kb region containing SBS-418 is reciprocal to that seen for the SBS-T4. SBS-418 is enriched in histone H3 methylation at Lys9 in wild-type thymocytes but is high in acetylation at H3 Lys9 and Lys14 in *Satb1*-null thymocytes (data not shown). Although ChIP studies must be done across the 150-kb SBS-418 genomic locus to delineate a discrete domain marked by specific histone modifications, the histone modification pattern at the SBS-418 site either in wild-type or *Satb1*-null chromatin is also consistent with the SATB1-dependent transcriptional status of the associated genes in each chromatin region. These data strongly suggest that SBSs serve as 'hot spots' for histone modification by binding to the SATB1 network, which consequently determines the fate of the epigenetic modification of defined regions.

All available evidence shows a tight correlation between tethering SBSs to the SATB1 network and regulation of genes whose expression is SATB1-dependent. Although a possibility formally exists that SATB1 may regulate expression of a transcription factor(s) that in turn regulates genes near SBSs, a direct cause–effect relationship between the two events is strongly suggested by the finding that SATB1 binding to an SBS *in vivo* causes important changes in the histone modification pattern, which is consistent with the expression status of the associated gene. In support of this concept, in the *Il2ra* locus, SATB1 recruits chromatin remodeling complexes to an SBS found in its intron, and this is correlated with proper expression of the gene and nucleosomal positioning over this locus[33]. Furthermore, the genomic sequences bound to SATB1 *in vivo* are anchored to the SATB1 network in wild-type thymocytes at the bases of chromatin loops. Thus, our data support a model in which chromatin organization by the SATB1 network is directly linked to gene regulation. It is noteworthy that those BURs identified as SBSs are highly conserved between mouse and human in their corresponding orthologous regions (data not shown).

In conclusion, our results on SATB1 suggest a new mechanism for cell type–specific gene regulation linking nuclear architecture, chromatin structure and functional organization of DNA sequences. Our data, together with our previous results[33], suggest that the SATB1 network may serve as a landing platform for chromatin-remodeling factors and can take the form of a complex intranuclear structure. This specialized structure can, in turn, allow factors to act on specialized genomic sequences that are also anchored to the network as bases of chromatin loops. The genomic sites bound to the SATB1 network seem to be hot spots for establishing a histone code. Biological consequences of these events may include proper expression, repression or inducibility of a discrete set of genes during T-cell development. Thus, the SATB1 network may provide a novel nuclear structure that functions as a tissue-specific organizer of DNA sequences.

Methods

Thymocyte activation. We isolated thymocytes from 2-wk-old wild-type and *Satb1*-knockout mice and resuspended them to a density of $1–2 \times 10^6$ cells ml^{-1} in RPMI medium containing 10% fetal bovine serum. For activation, we added 10 ng ml^{-1} PMA and 500 ng ml^{-1} ionomycin to the culture medium and cultured thymocytes at 37 °C for 0–4 h. We isolated total RNA with Trizol reagent (Sigma). Mice were treated according to Lawrence Berkeley National Laboratory's Animal Care and Use Program, which is accredited by American Association for the Accreditation of Laboratory Animal Care.

RNase protection and RT–PCR assays. We carried out RNase protection assays using 20 μg RNA per reaction and using Riboquant Kit (BD Pharmingen) according to the manufacturer's protocol. We used the mouse *Myc* multi-probe template (BD Pharmingen) to prepare ^{32}P-

labeled RNA antisense probes, which were transcribed using T7 polymerase and the *in vitro* transcription kit (BD Pharmingen). The *Zap70* cDNA fragment cloned in Bluescript for antisense RNA has been previously described[27]. We cloned the probe 418-22 into pAMP10 for RNase protection. Primer sequences are available on request. We generated the antisense riboprobes for 418-22 and *Zap70* using the *in vitro* transcription kit to give the free probe length of 429 bp and 440 bp, respectively. It contained 255 bases of 418-22 sequence and 410 bases of *Zap70* sequence. We analyzed the ^{32}P-labeled RNA duplexes by electrophoresis in 6% (w/v) polyacrylamide/8 M urea gels. For RT–PCR, we reverse-transcribed total RNAs from thymus and brain tissue of *Satb1*-knockout and wild-type mice into single-stranded cDNA using Superscript II RNase H$^-$ reverse transcriptase (Invitrogen) as described in manufacturer's manual. We carried out PCR reactions in a total volume of 25 μl containing cDNA made from 0.2 μg of total RNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM of primers corresponding to each gene sequence to be amplified and 1.25 U of Amplitaq Gold DNA polymerase (Applied Biosystems). Primer sequences and product sizes are available on request. The amplification was carried out for 25 cycles except for mouse homolog of *EEF1G* (20 cycles) and 418-22 (42 cycles). Each cycle consisted of the following steps, using GeneAmp PCR system 9700 (Perkin Elmer): 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s. We separated PCR products by electrophoresis on a 2% agarose gel and visualized them by staining with ethidium bromide.

To determine which ESTs represent independent transcripts in the UniGene cluster (Mm.24739), we carried out RT–PCR using different sets of primers, including the primer set 15 in ChIP assay (shown in Fig. 6) overlapping AU051681 and BC025574. These primer sequences are available on request. Only those primers designed within each EST showed bands of the expected sizes by RT–PCR. None of the 3′ primers designed from the three ESTs (AU051681, BC025574 and AA274079) generated RT–PCR bands with a 5′ primer designed from the mouse homolog (BC018474) of human *KIAA0766*.

ChIP assays to determine SBS and histone modification. We cloned *in vivo* SBSs from mouse thymocytes following the procedure previously described[35]. Main steps involved are the following: (i) formaldehyde crosslinking of chromatin in thymocytes, (ii) purification of cross-linked genomic DNA from free proteins by urea-gradient centrifugation, (iii) restriction digestion of protein-crosslinked genomic DNA with *Sau*3A, (iv) immunoprecipitation of SATB1-crosslinked genomic DNA fragments with antibody against SATB1, (v) ligation-mediated PCR amplification and cloning of the SATB1-bound DNA and (vi) sequencing of the cloned DNA. To determine if any given sequence is bound to SATB1 *in vivo*, restriction-digested crosslinked chromatin from step (iii) above was pre-cleared by incubation first with protein A–Sepharose 4B beads alone and then with non-immune rabbit serum and protein A–Sepharose beads. We divided the pre-cleared chromatin and incubated it overnight with either preimmune serum or antibody against SATB1. We washed complexes on beads four times with 1.0% Nonidet P-40 in phosphate-buffered saline and two times with washing buffer (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA). We then digested the samples with 250 μg ml^{-1} proteinase K, treated them at 68 °C for 6 h to revert crosslinking and subjected them to phenol-chloroform extraction before ethanol precipitation with glycogen. We amplified by PCR DNA sequences from each immunoprecipitated chromatin pool and from non-immunoprecipitated chromatin (whole-cell extract) with Amplitaq Gold (Applied Biosystems). Primer sequences used for *Myc*, SBS-336 and *Zap70* are available on request. For ChIP to determine histone modification pattern, we used antibodies to methylated H3 Lys9, to methylated H3 Lys4 and to acetylated H3 Lys9/Lys14 (Upstate Biotechnology) for immunoprecipitation. We did PCR using 1 cycle of 95 °C for 10 min and 30 cycles of 95 °C for 45 s, 52 °C for 45 s, 72 °C for 1 min. We labeled PCR products by including 0.25 μl [α-^{32}P] dCTP (10 mCi ml^{-1}) in each reaction. We used the primers from the *Zap70* locus as internal control. Primer sequences are available on request. We resolved PCR products by electrophoresis on 4% polyacrylamide gel and quantified them using Storm phosphoimager (Amersham Biosciences). We quantified these results by calculating relative precipitated enrichments of P1-T4 sequences between ChIP DNA and whole-cell extracts as previously described[9] and plotting data in alignment with the map of the P1-T4 locus.

Amplified FISH. We prepared nuclei, halos and nuclear matrix for FISH analysis exactly as described[36]. We carried out tyramide-amplified FISH with TSA indirect (ISH) kit (Perkin Elmer Life Science) with modifications as described[36]. We prepared the 700-bp probe containing the SBS upstream of *Myc* by PCR amplification of mouse genomic DNA. Primer sequences for this PCR are available on request.

Immuno-FISH labeling. We carried out immuno-FISH essentially as described[44] with modification. We incubated thymocytes fixed with paraformaldehyde with antibody against SATB1 (1:300; ref. 14) and goat antibody against rabbit IgG conjugated with Alexa-594 according to standard immunofluorescence protocol. We then post-fixed cells with 4% paraformaldehyde to preserve proteins detected by antibody. To detect the *Myc* signal, we carried out amplified FISH as described above.

Immunostaining and image analysis. We prepared thymocytes from 2-wk-old mice and fixed them in 4% paraformaldehyde. We immunostained the cells with rabbit polyclonal antibody against SATB1 1583 (1:300; ref. 14) and then incubated them with goat antibody against rabbit conjugated with Alexa-594 (1:400; Molecular Probes). Alternatively, we stained thymocytes with rat monoclonal antibody against M31 (ref. 50; Serotec) and then incubated them with goat antibody against rat conjugated with Alexa-488. We stained DNA with DAPI (Sigma). The images were collected by a DeltaVision microscope according to the manufacturer's instruction (Applied Precision). We collected optical sections at 0.2-μm Z-spacing with a 100× N.A. 1.35 lens using a wide-field optical sectioning microscope, and we deconvolved three-dimensional image stacks using an iterative point-spread function using software provided by the manufacturer (SoftWoRx, Applied Precision). We generated projections using a maximum-intensity algorithm and pseudocolored them. We did further scaling and image processing using Adobe Photoshop.

URL. The tissue-specific expression of *KIAA1598* is shown in http://www.kazusa.or.jp.

Accession numbers. *KIAA1598*, AB046818; mouse homolog of *KIAA1598*, BC030338; *SATB1*, NM_002971; *Satb1*, U05252; EEF1G, P26641; mouse homolog of *EEF1G*, AK007869; *Myc*, M26861; mouse *Mlh1*, AF250844; *KIAA0766*, NM_014805; mouse homolog of *KIAA0766*, BC018474; genomic sequences containing BAC-418, NT_039692; P1-T4, NT_039480; BAC-439, AC023608.

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Competing interests statement

The authors declare that they have no competing financial interests.

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