SATB1 packages densely-looped, transcriptionally-active chromatin for coordinated expression of cytokine genes

Shutao Cai, Charles C. Lee, Terumi Kohwi-Shigematsu[#]

Lawrence Berkeley Laboratory, University of California,

Berkeley, CA 94720

Corresponding author:

Terumi Kohwi-Shigematsu, Ph. D.

Senior Scientist

Lawrence Berkeley Laboratory

1 Cyclotron Rd (mail stop 84-171)

University of California

Berkeley, CA 94720

TEL 510-486-4983

FAX 510-486-4545

email terumiks@lbl.gov

SUMMARY

SATB1 is an important regulator of nuclear architecture that anchors specialized DNA sequences onto its cage-like network and recruits chromatin remodeling/modifying factors to control gene transcription. We studied the role of SATB1 in regulating the coordinated expression of *Il5, Il4,* and *Il13* from the 200kb cytokine gene cluster region of mouse chromosome 11 during T-helper 2 (Th2)-cell activation. We show that upon cell activation, SATB1 is rapidly induced to form a unique transcriptionally-active chromatin structure that includes the cytokine gene region. Chromatin is folded into numerous small loops all anchored by SATB1, is histone H3 acetylated at lysine 9/14, and associated with Th2-specific factors, GATA3, STAT6, c-Maf, the chromatin-remodeling enzyme Brg-1, and RNA polymerase II across the 200kb region. Before activation, the chromatin displays some of these features, such as association with GATA3 and STAT6, but these were insufficient for cytokine gene expression. Using RNA interference (RNAi), we show that upon cell activation, SATB1 is not only required for chromatin folding into dense loops, but also for c-Maf induction and subsequently for *Il4, Il5,* and *Il13* transcription. Our results show that SATB1 is an important determinant for chromatin architecture that constitutes a novel higher-order, transcriptionally-active chromatin structure upon Th2-cell activation.

INTRODUCTION

Cells from different tissues require different programs of gene expression to produce proteins required for their particular function. Chromatin structure, which is believed to be an important determinant of gene activation or silencing, is regulated by various epigenetic processes[1-3]. Recent studies have also shown that the spatial organization of chromatin in the nuclei is an important component of gene regulation. The recently developed chromosome conformation capture (3C) assay[4] has revealed that chromatin looping events, which bring distal DNA sequences into close proximity with each other, correlate with gene expression levels at several loci, including the *β-globin* locus[5-8], the *Igf2-H19* locus[9], the cytokine gene cluster region[10,11], the *immunoglobulin kappa* gene locus[12], and the *Dlx5/Dlx6* locus[13].

To understand the mechanisms that underlie tissue-specific gene regulation in the context of the three-dimensional chromatin environment, it is essential to identify the factors that

mediate chromatin folding. One factor, SATB1, has been shown to function as a cell-type-specific 'genomic organizer.' SATB1 is expressed predominantly and at high levels in thymocytes, but its expression is reduced or undetectable in unactivated mature T cells[14,15]. SATB1 exhibits a 'cage-like' nuclear distribution in thymocytes surrounding heterochromatin and anchors chromatin at specialized DNA sequences to keep it in a folded conformation[16,17]. SATB1 targets chromatin remodeling complexes to the anchored sites, and thereby regulates the status of histone modification and nucleosomal positioning over long-distance DNA sequences[18]. The anchored sequences have a characteristic 'ATC sequence context, ' which is enriched in stretches of DNA sequences containing a mixture of As, Ts, and Cs, but no Gs, on one strand[14]. Such genomic sequences have an exceptionally highly propensity to become base unpaired when subjected to superhelical strain and are referred to as a base-unpairing region (BUR)[19]. SATB1 was originally cloned as a protein that specifically recognizes BURs in double-stranded, but not in single-stranded, DNA. When BURs are mutated to disrupt the ATC sequence context to abrogate the 'base-unpairing' propensity, SATB1 no longer binds[14].

Hundreds of anchored loci have been cloned, and the expression levels and patterns of a large number of genes in close proximity to these loci have been shown to be disrupted in SATB1-deficient thymocytes[15,17]. Furthermore, T cell development is arrested mainly at CD4$^+$/CD8$^+$ (double-positive) stage [15]. SATB1 and its homolog SATB2 are expressed in multiple, but restricted cell types[20-24], and some important post-translational modifications have been characterized for these proteins[25,26].

In addition to SATB1, methyl CpG-binding protein 2 (MeCP2) was recently found to regulate higher-order chromatin organization[13]. MeCP2 has been reported to be a repressor of gene transcription[27,28], and *MeCP2* mutations are associated with Rett Syndrome, a neurodevelopmental disorder[29]. MeCP2 has been recently shown to be responsible for folding into a loop transcriptionally silent chromatin at the locus of one of its target gene genes, *Dlx5/6*[13]. In the brains of MeCP2-null mice (a mouse model for Rett syndrome), only chromatin loops derived from transcriptionally active chromatin are formed, rather than loops associated with silent chromatin, resulting in upregulation of *Dlx5* and *Dlx6* transcription.

The cytokine gene cluster located on mouse chromosome 11 has been studied extensively as a model system of the relationship between chromatin organization and gene expression[30-32]. Upon antigen stimulation, naive CD4$^+$ T helper cells differentiate into two subsets of T helper

cells (Th1 cells and Th2 cells)[33-35], which are distinguished by their specific functions and patterns of cytokine expression. Th1 cells produce inflammatory cytokines, such as TNF-β and gamma interferon (IFN-γ), and increase cellular immunity mediated by macrophages. In contrast, Th2 cells produce a different group of cytokines (*Il4, Il5. Il6, Il10*, and *Il13*), which help B cells secrete antibodies. In response to T-cell receptor (TCR) stimulation, cultured naïve CD4$^+$ (T-helper) cells undergo differentiation into either Th1 or Th2 cells, depending upon the presence of IL-12 or IL-4, respectively, in the extracellular environment. This differentiation is mediated by signaling proteins that include STAT4- and STAT6, and results in expression of specific transcription factors such as GATA-3 and c-Maf by Th2 cells, and of T-bet by Th1 cells. Epigenetic changes such as histone hyperacetylation[36-39], DNA demethylation[40,41] and the development of specific DNAse I hypersensitive sites are also associated with T-helper cell differentiation[42-46].

Genes that encode the Th2 cytokines Il4*,* Il5 and Il13 are clustered on a 120 kb region of mouse chromosome 11, and their expression is coordinately regulated. Coordinate regulation of these cytokine genes in a gene cluster could involve higher-order chromatin structure formation. In fact, long-range intrachromosomal interactions between the promoters of these genes which bring them into close spatial proximity has been reported[10]. This type of 'chromatin core' conformation has been detected in both T cells, B cells, as well as non-lymphoid cells. The participation of the Th2 locus control region (LCR) in this conformation was found to exist in a more cell-type restricted manner, identified in CD4$^+$ T cells and natural killer cells, but not B cells or fibroblasts. LCRs are long-range regulatory regions that can confer tissue-specific enhancer activity and copy-number-dependent expression in linked genes[47-49]. A LCR was identified as a 25 kb stretch of DNA within the 3' half of the >50kb *Rad50* gene located between *Il5* and *Il13*[45]. Among Th2-specific DNase-I hypersensitive sites in the LCR, *Rad50* hypersensitive site 7 (RHS7) was found to be important for long-range intrachromosomal interactions between the LCR and cytokine gene promoters and for regulation of cytokine gene expression[50]. More recently, interchromosomal interaction between the promoter region of the IFN-γ gene on chromosome 10 and the regulatory region of the Th2 cytokine locus on chromosome 11 was reported. This interchromosomal interaction was reportedly lost during the differentiation of naïve T cells into effector T-helper cells[11].

4

We investigated the three-dimensional transcriptionally active chromatin structurethat is formed by the 200kb cytokine gene cluster region by comparing chromatin loop organization in pre-activated and activated Th2 cells. We show that upon Th2 cell activation, the rapid induction of SATB1 is required for expression of *Il4, Il5* and *Il3*. Using the recently devised chromatin immunoprecipitation (ChIP)-loop assay, we analyzed SATB1-associated chromatin loops. We identified a transcriptionally-active chromatin configuration that is characterized by dense-loopingand fastened by SATB1 at its base. Furthermore, we show that the formation of this transcriptionally active chromatin structure requires SATB1.

RESULTS

Expression of SATB1 upon activation of Th2 cells

We examined the role of SATB1 in T cells using the D10.G4.1 conalbumin-specific Th2 (D10) cell line. Upon activation by conconavalin A (Con A), these cells express Th2-specific cytokines including *Il4, Il5, Il10, Il13,* as confirmed by an RNAase-protection assay (Fig. 1a). Although the genes *Kif3a* and *Rad50* are located in close proximity to *Il4, Il5* and *Il13* and reside in the same gene cluster region, their expression is not inducible, but rather are constitutively expressed at very low levels in these cells. Immunostaining of D10 cells with a rabbit polyclonal anti-SATB1 antibody (1583) showed that although SATB1 was hardly detectable prior to T-helper cell activation, it was induced rapidly within 30 minutes after activation. In the nucleus, it exhibited a dense three-dimensional cage-like distribution surrounding heterochromatin (Fig. 1b). This distribution of SATB1 is similar to that observed in previous studies with thymocytes[17]. In contrast to SATB1, whose expression is induced upon activation, the Th2 cell-specific transcription factor GATA3 is highly expressed in both resting and activated D10 cells (Fig. 1b). In activated D10 cells, GATA3 is largely co-localized with SATB1 (Fig. 1b).

Identification of SATB1-bound sequences in the cytokine gene cluster

SATB1 is known to specifically bind BUR sequences *in vitro* and *in vivo* in thymocytes and Jurkat lymphoblastic cells [16,17]. Therefore, we tested whether the SATB1 cage-like structure could fold chromatin across the cytokine gene cluster region by anchoring BURs within the cluster. To this end, we identified a total of nine SATB1-bound sequences (SBSs) from two BAC clones encompassing the 200 kb region that contains *Il5, Rad50, Il13, Il4* and *Kif3a* (Fig 2,

indicated by vertical arrows, SBS-C1 to SBS-C9). These nine SBSs were cloned by isolating glutathione-S-transferase (GST)-SATB1-bound *Sau*3AI digested fragments from each of the two BAC clones, using protein-G sepharose beads, followed by ligation-mediated PCR amplification (LM-PCR) and sequencing. In this 200kb region, we verified by gel shift assays that there are no additional sequences that bound SATB1 *in vitro*.

To examine whether each of the nine SBSs in the 200kb region (SBS-C1 to SBS-C9) bind to SATB1 *in vivo*, we performed chromatin immunoprecipitation using urea-gradient purified crosslinked chromatin (urea-ChIP assay) that was digested with Sau3A[51]. We found that all nine SBSs identified *in vitro* were immunoprecipitated specifically by the anti-SATB1 antibody from the pools of *Sau*3A-digested chromatin fragments isolated from formaldehyde-crosslinked activated D10 cells (Fig 2). The control DNA fragments, which did not have any potential SATB1-target sequences, were undetectable in urea-ChIP DNA prepared using anti-SATB1 antibody, under the same PCR amplification condition (Fig. 2). In addition to the nine SBSs, conserved nucleotide sequence-1 (CNS-1) and CNS-2 were found also associated with SATB1. CNS-1 and -2 are the two most prominent conserved sequences in the cytokine gene cluster region and they contain Th2-specific DNase 1 hypersensitive sites[52]. The association between SATB1 and CNS-1/-2 was surprising, as these sequences do not contain a SATB1 binding site. However, CNS-1 and –2 sequences typically appeared after more PCR cycles than the SBSs (data not shown), suggesting that SATB1 may bind at the CNS-1/-2 sites more weakly and/or indirectly through other factors. This is what has been observed to occur at the murine *Il2Ra* locus, where indirect binding of SATB1 to the *Il2Ra* promoter was detected *in vivo*[18]. Crosslinked chromatin fragments immunoprecipitated with pre-immune serum (as a control) did not produce any PCR products, under the same PCR condition. As expected, no PCR signals were generated by the urea-ChIP assay when resting D10 cell samples were treated with the anti-SATB1 antibody, presumably due to the absence of SATB1 in these cells (Fig. 2). These data demonstrate that SATB1 binds to nine SBSs, as well as to CNS-1 and –2, *in vivo* in activated D10 cells.

Chromosome conformation capture and urea-ChIP-loop assay

We next asked whether SBSs are involved in higher-order packaging of chromatin at the cytokine cluster region upon Th2 cell activation. Chromatin loops that form *in vivo* can be

'trapped' during formaldehyde crosslinking of cells. If remote DNA sequences are physically brought in close proximity by chromatin looping *in vivo*, they can be found in the same crosslinked chromatin fragment, and the DNA sequences at the stem of chromatin loops can be identified. Briefly, after digesting crosslinked chromatin with a restriction enzyme that removes the loop portion of chromatin, the two sequences initially located at the stem of loop can then be ligated intra-molecularly, after dilution of the chromatin sample to avoid inter-molecular ligation events. After reverse crosslinking (to remove proteins), purified genomic DNA that had been intra-molecularly ligated, excluding the loop portion of DNA, can be amplified by PCR, using primer pairs designed from the two distal sequences of interest. The ligation products detected by PCR indicates physical interactions between DNA fragments held together by higher-order chromatin structure. This method called the chromosome conformation capture (3C) assay[4] allows us to detect the frequency with which two remote genomic sequences interact in space in a given nucleus. The crosslinking frequencies of any two DNA fragments, as determined by the intensity of the PCR signals of a ligation product, depends on the relative proximity of the two DNA fragments to each other at a given time point. Relative ligation crosslinking frequencies of any two DNA fragments were calculated as described (supplementary Fig. 1a-c) to normalize various parameters such as PCR amplification efficiencies, ligation and crosslinking efficiencies, the amounts of the template initially used.

We studied chromatin looping employing both the 3C assay[4], as well as the more recently devised ChIP-loop assay[13] (illustrated in supplementary Fig. 1a-c). In the latter assay, the 3C analysis is performed specifically on the chromatin fragments that were immunoprecipitated with an antibody specific for a chromatin associated protein of interest, such as SATB1. Although both assays allow for the examination of long-range DNA interactions between regulatory elements, in the context of chromatin spatial organization, the advantage of the ChIP-loop assay is that it enables us to study a specific group of chromatin loops that are fastened at their base with a specific protein, or that associates with specific histone modification. We examined a total of 20 Sau3A fragments within which we designed forward and reverse primers to screen for chromatin loops, including sites corresponding to all nine SBSs, CNS-1, CNS-2, promoter regions and other intergenic sequences.

Chromatin loop structure changes upon T-helper cell activation

Using the 3C assay, we first examined chromatin looping in the 200 kb cytokine gene cluster, using total chromatin isolated from resting D10 cells. We initially analyzed the farthest-spaced SBSs (SBS-C1 and SBS-C9), which are separated by 170 kb. The 3C assay revealed that SBS-C1 was spatially juxtaposed to CNS-2 (using primer 15), and SBS-C7 (using primer 16) (Fig. 3). Because SBS-C1 is only 0.8 kb away from the *Il5* promoter (primer 1), a PCR-amplified product from these two sites was inevitably generated. In resting D10 cells, a very low crosslinking frequency was detected between SBS-C1 (primer 2) and SBS-C9 (primer 20), as indicated by a faint PCR band of the ligation product. Using SBS-C9 as a reference point, we found that SBS-C9 is spatially linked to the *Il5* promoter (primer 1). SBS-C9 also interacts with SBS-C2 (primer 4) and a portion of the LCR (primer 10, which resides immediately adjacent to RHS7[46,50], and primer 11, which are separated by 4kb). All other primer combinations did not generate ligation products even after much increased PCR cycles (see Materials and Methods). These data indicate the presence of specific three-dimensional loops that involve two distal SBSs (SBS-C1 and SBS-C9) in resting D10 cells.

To study chromatin organization in activated D10 cells, we employed both the 3C assay without immunoprecipitation and the ChIP-loop assay in which we used chromatin fragments immunoprecipitated by the anti-SATB1 antibody. We found that after T-cell activation, chromatin in this 200kb region undergoes a major change in three-dimensional chromatin organization. This was revealed by the appearance of many additional ligation products when chromatin was analyzed using both the 3C assay and the ChIP-loop assay. By DNA sequencing, we confirmed that these products were derived from ligation of two distal chromatin fragments of the 200 kb cytokine gene locus. Interestingly, in activated D10 cells, the loop organization that was derived specifically from SATB1-associated chromatin was strikingly similar to that obtained by the 3C assay, which examines the entire chromatin pool. In fact, all bands detected in the 3C assay were also generated in the ChIP-loop assay. The ChIP-loop assay, in comparison to the 3C assay, however, revealed several additional ligation products, as well as increased crosslinking frequencies of some common products. The identification of these additional ligation products is most likely due to the enrichment of the SATB1-bound templates in the ChIP-loop assay (see Materials and Methods). Our results strongly suggest that in activated D10 cells, the chromatin loops that form in the 200kb region are all bound to SATB1 at their bases.

The ChIP-loop data show that when D10 cells become activated, SBS-C1 makes contacts with all four intronic SBSs of *Rad50* (SBS-C3, -C4, -C5, and -C6; using fragments 6-9), of the *Il13* promoter (using fragments12), of CNS-1 (using fragment13), and strengthens its interaction with the *Il4* promoter (using primer 14) and CNS-C9 (using fragment 20) (Fig. 3). In particular, the crosslinking frequency between SBS-C1 and SBS-C9 was increased nearly 10-fold after D10 cell activation, from a frequency of less than 0.5 to almost 5.0 (Fig. 3). Using SBS-C9 as a reference point, new contacts with all four *Rad50* intronic SBSs (fragments 6-9), as well as with the *Il13* and *Il4* promoters (fragments 12 and 14) were observed upon D10 cell activation. In both resting and activated cells, SBS-C1 remains associated with CNS-2 (fragment 15) and SBS-C7 (fragment 16), and SBS-C9 interacts with LCR (fragments10,11).

Although SBS-C1 and SBS-C9 interact directly with each other, each of these two sequences shows individual differences in crosslinking frequency with other sites. For example, SBS-C9 shows much stronger crosslinking frequency to both *Il13* and *Il4* promoters, as well as with CNS-1, than does SBS-C1. Furthermore, the 3'LCR (fragments 10 and 11) can be ligated to SBS-C9, but not to SBS-C1, in both resting and activated cells. We speculate that the intramolecular ligation step could be hampered in crosslinked chromatin, depending on the spatial orientation of the two closely located Sau3A restriction sites. The overall results, using SBS-C1 and SBS-C9 as reference points, indicate that when D10 cells are activated, chromatin in the 200 kb cytokine cluster region is folded into smaller loops that involve remote sequences.

SATB1-bound sequences in *Rad50* introns form chromatin loops

Of the nine SBSs that lie within the 200kb cytokine gene region, four are clustered within the introns of *Rad50*, a repair gene that is constitutively expressed at very low levels. Although expression of *Rad50* is independent of the coordinately regulated expression of the cytokine genes in the 200kb region, the large *Rad50* gene locus contains the LCR containing multiple DNase I hypersensitive sites specific for Th2 cells [45,46]. The enrichment of SBSs in *Rad50* strongly suggests that this gene locus has a central role in chromatin organization at the cytokine gene cluster. We further examined this possibility using *Rad50* intronic SBSs as fixed reference points.

Some chromatin loops derived from *Rad50* SBSs were identified in resting D10 cells. The *Il5, Il4,* and *Il13* promoters have very low to moderate crosslinking frequency with some of

these *Rad50* SBSs. For example, we found the *Il5* promoter (primer 1) linked to SBS-C3, SBS-C4 and SBS-C5, the *Il4* promoter (fragment 14) linked to SBS-C4, SBS-C5 and SBS-C6, and the *Il13* promoter (fragment 12) linked to SBS-C5 and SBS-C6 (with a greater preference in crosslinking for the latter). SBS-C6, which resides within the LCR, is juxtaposed to the *Il-13* promoter (fragment 12), CNS-1 (fragment 13) and 3' end of LCR near RHS7 (fragment 10). We also observed a PCR-amplified product between SBS-C4 and SBS-C5. However, since they are only 1.25kb apart, a ligation product would inevitably be produced in our assays even without looping, and their individual looping events with remote sequences would appear to be similar. Typically in our hands, the two sequences have to be at least 4 kb apart in order to assess independent looping events.

In stark contrast to resting cells, when D10 cells were activated, we observed a dramatic re-organization of chromatin containing the intronic regions of *Rad50* (Fig. 4a-d). Both the 3C and ChIP-loop assays revealed strikingly similar crosslinking patterns between each of the *Rad50* SBSs and remote sequences. Upon activation, ligation products between any primer combination among these *Rad50* SBS fragments appeared, indicating that these SBSs are all brought into spatial close proximity, comprising a single core base that is bound to SATB1. This is indicated by the appearance of new ligation products by PCR in activated D10 cells, when each of the *Rad50* intronic SBS fragments is used as a fixed reference point in both 3C and ChIP loop assays, compared to resting cells (Fig. 4a-d). The interaction of *Rad50* intronic SBSs with other gene promoters is also strengthened upon D10 cell activation. In contrast to the dynamic looping patterns exhibited by the *Rad50* intronic SBSs as observed before and after activation, the fragment 10, located within the LCR, remained connected to SBS-C9 (primer 20), the *Il4* and *Il13* promoters (primers 14 and 12) and SBS-C6 (fragment 9). These interactions did not vary significantly between the resting or active state of the D10 cells (Fig. 4e), suggesting that the chromatin configuration represented this set of loops might serve as the chromatin backbone structure in this 200 kb region.

Looping between promoters and regulatory sequences increases upon D10 cell activation

We next examined the spatial interactions between the *Il4, Il5, Il13* and *Rad50* promoters and their regulatory sequences using sequences in these four promoter sites as fixed reference points in the loop assays. In resting cells, in addition to the low to moderate-level crosslinking of

the *Il5, Il13*, and *Il4* promoters with *Rad50* intronic SBSs (fragments 6-9) (Fig. 4), the *Il5* promoter contacts the SBS-C7 (fragment 16) (Fig. 5a). All three promoters make contact with CNS2 (fragment 15)(Fig. 5a,c,d). Weak interactions between the *Il4* promoter and the *Il13* promoter (fragment 12), as well as between the *Il4* promoter and the *Il5* promoter (fragment 1), have been reported, but no ligation product was found to form between primers from DNA fragments corresponding to the *Il5* and *Il13* promoters (Fig. 5a, c, d). This might be caused by the spatial orientation of digested ends of crosslinked chromatin, which may not be compatible for intra-molecular ligation and would result in the absence of the PCR product between the *Il5* and *Il13* promoters.

Upon D10 cell activation, the *Il5* promoter makes new and strong connections with the *Il13* and *Il4* promoters (fragments 12 and fragment 14, respectively) (Fig. 5a). This is supported by data shown in Fig. 5c,d using *Il13* ad *Il4* as fixed reference points as well. In addition, the frequency of crosslinking between the *Il5* promoter and CNS-1 (fragment 13) is also increased upon D10 cell activation (Fig. 5a). The *Rad50* promoter, however, does not participate in any looping upon D10 cell activation (Fig. 5b). This is consistent with the fact that *Rad50* expression is not induced upon D10 cell activation (Fig. 1). The region between the *Rad50* promoter (fragment 3) and SBS-C2 (fragment 4) was amplified in all reactions, because the primers from these DNA fragments are only 1.25kb apart. This PCR product was not immunoprecipitated in the ChIP-loop assay. Taken together, when D10 cells become activated, the *Il4, Il5* and *Il13* promoter sequences become tightly associated in close spatial proximity, and the distal SBSs such as SBS-C1 and SBS-C9, as well as intronic *Rad50* SBS, become clustered (Fig 4 and 5). Because these sites are mutually linked and chromatin loops can be detected in SATB1-associated chromatin fragments (ChIP-loop assay), these loops are assembled with SATB1 at their base.

GATA3 and STAT6 bind DNA throughout the 200 kb cytokine region

In order to gain insight into the biological significance of the SATB1-associated dense loop structure for gene expression, we explored the binding status of the two Th2 cell-specific transcription factors, GATA3 and STAT6, in the 200 kb locus before and after D10 cell activation. Both GATA3 and STAT6 are required for the Th2 cell–specific lineage commitment[53-56]. We performed real time PCR, using the same 24 primer sets as shown in Fig.

2, on urea gradient-purified, crosslinked chromatin fragments (see Fig. 2). We found that GATA3 is bound to multiple sites within the 200 kb region in resting D10 cells, including all promoters and most of the SBSs (Fig. 6a). The binding pattern of GATA3 in this region is not significantly different between resting and activated D10 cells, except that GATA3 binding to the *Il13* promoter, CNS-1 and the *Il4* promoters increased upon cell activation. In a similar manner to GATA-3, the transcription factor STAT6 was bound to multiple positions throughout the 200kb region in resting D10 cells (Fig. 6b). However, its level of binding at SBS-6 an SBSC7 increased upon cell activation.

c-Maf and RNA polymerase II are recruited to the 200 kb region upon activation

Using our real time PCR analysis of urea-ChIP chromatin, we also searched for transcription factors that bound to the 200kb locus specifically upon T-helper cell activation. c-Maf, a transcription factor that is specifically expressed in Th2 cells, is induced upon T-helper cell activation, which mediates *Il4* expression[57,58]. Our analysis showed that the c-Maf protein is recruited to the 200kb region after D10 cell activation. In activated cells, c-Maf binding was detected at all sites examined in the200 kb region, except for R2 (primer set 10) and R5 (primer set 14) (Fig. 6c). This broad-spectrum binding pattern of c-Maf was unexpected, because of its previously characterized specific regulation of *Il4* gene expression and its *in vivo* binding to the *Il4* promoter. We observed that c-Maf bound strongly to the *Il4* promoter region (primer sets 17 and 18), to the *Il5* and *Il13* promoters, and also to all SBSs.

We next examined the binding of RNA polymerase II in this region, to determine whether it binds to DNA before or only after T-helper cell activation. We used an antibody against RNA polymerase II that detects all forms of the enzyme. Importantly, we found that the 200 kb locus becomes primarily associated with RNA polymerase only after D10 cell activation Like c-Maf, RNA polymerase II binding bound multiple positions throughout the 200kb region (Fig. 6d), and there is a significant similarity in the preferential binding sites between c-Maf and RNA polymerase II. Upon cell activation, both factors were tightly bound to the R3 region (primer set 12), the *Il4* promoter region (primer sets 17 and 18), SBS-C7 and SBS-C8. We used immunostaining analysis to determine the cellular distribution of RNA polymerase II, in both resting and activated D10 cells (supplementary Fig. 2). Although the overall level of RNA polymerase II bound to the 200kb region is dramatically increased following D10 cell activation,

there was no major difference in cellular levels of the protein before and after activation This result was also verified by Western analysis (data not shown), indicating that RNA polymerase II was recruited to the 200kb locus only after T-helper cell activation, even though it was already present in resting cells. Upon cell activation, we detected an extensive co-localization in the nuclear localization of RNA polymerase II, c-Maf and Brg-1 with SATB1and GATA3 (supplementary Fig. 2).

Elevated histone H3 K9/14 acetylation and Brg-1 association with the 200kb region upon activation

In resting cells, we found the ATP-dependent chromatin remodeling enzyme Brg1 bound to the transcriptionally silent chromatin the cytokine gene cluster region. Upon cell activation, compared to the resting state, our real time PCR data show that the binding of Brg-1 increased significantly by 2-10 fold at many positions within the 200kb region, such as at SBS-C2 through SBS-C5, CNS1, CNS-2, and at all promoters (Fig. 6e).

In transcriptionally active chromatin, histone H3 is acetylated at lysines 9 and 14 (K9/14)[1]. We examined the histone acetylation status at the cytokine gene locus in D10 cells before and after activation, using a urea-ChIP assay followed by real time PCR with an antibody against acetylated histone H3 at K9/14. We found a dramatic increase in the level of histone H3 acetylation after cells were activated by Con A (Fig. 6f, red line), especially at SBS-C6. It is important to note that before activation, all sites examined within the 200kb region already had some histone H3 acetylated at K9/14, as shown separately (Fig. 6f, blue line). Before cell activation, none of the 24 sites were associated with histone H3 at methylated lysine 9, however, which is a characteristic of silent chromatin (data not shown). Therefore, although we observed a dramatic increase in histone H3 K9/14 acetylation upon activation, indicating an increased number of histone H3 acetylated at K9/14 , this locus was acetylated to some extent prior to activation.

SATB1 is required for chromatin reorganization and cytokine expression

Using an RNAi strategy[59], we examined whether SATB1 is necessary for induction of *Il4, Il5*, and *Il13* expression upon D10 cell activation. D10 cells were stably transfected with a construct that expresses short hairpin RNA (shRNA)[60] against *Satb1* (D10-*Satb1* shRNA cells*)*.

When these cells were activated with Con A, SATB1 was found to be expressed at less than 10% of that in wild-type D10 cells (Fig. 7a). Total RNA was isolated from D10-*Satb1* shRNA cells and was analyzed in an RNase protection assay. We found that the mRNA transcripts for *Il4, Il5, Il13* were either absent or were substantially reduced in the D10-*Satb1*-shRNA cells, even though the levels of other transcripts, such as *I32* and *Gapdh* RNA, were unaltered (Fig. 7b). The effects of SATB1 shRNA were most pronounced on *Il4* levels;no *Il4* transcript was detected in D10-*Satb1* shRNA cells. In addition to this effect on *Il4* expression*, Il5* and *Il13* transcripts were also greatly reduced in D10-*Satb1*-shRNA cells, indicating that SATB1 is necessary for the coordinate transcription of *Il5, Il4,* and *Il13* genes upon D10 cell activation.

We tested whether the absence of the *Il4* transcript in Con A-treated D10-*Satb1*-shRNA cells might be due to the lack of inducibility of c-Maf in the absence of SATB1. Immunostaining analysis of c-Maf protein levels after cell activation revealed that this was true; c-Maf was not induced in activated T-helper cells (Fig. 7c). We conclude that SATB1 is necessary for induction of c-Maf and the subsequent activation of *Il4* transcription.

We hypothesized that SATB1 might be required for 'new loop formation' associated with the transcriptionally active chromatin. Using the 3 C assay, we analyzed the loops that emanated from SBS-C1 and SBS-C9 in both wild-type and D10-*Satb1* shRNA cells. We found that SATB1 is indeed required for the loop formation upon activation Using SBS-C1 and SBS-C9 as fixed reference points, the loop patterns that formed in chromatin from the D10-*Satb1* shRNA cells were very similar to those observed in unactivated wild-type cells(Fig. 8a,b). These results show that SATB1 is necessary for folding chromatin into a three-dimensional active chromatin configuration.

DISCUSSION

We describe a SATB1-dependent, transcriptionally-active chromatin structure that occurs in the cytokine gene cluster region of mouse chromosome 11 upon activation of Th2 cells. We show that SATB1, which is rapidly induced upon Th2 cell activation, is required for folding chromatin into numerous small-size loops across the 200kb cytokine gene region, induction of c-Maf protein, and transcription of *Il4*, *Il5*, and *Il13*.

Previous studies using 3C assays on the cytokine gene cluster region covered a 120kb region that included *Il5, Rad50, Il4* and *Il13*[10]. These studies analyzed three-dimensional

chromatin structure formed by this region in various cell types and detected a chromatin configuration called a 'poised state' that is restricted to T helper cells and NK cells. In the present study, we introduced SATB1 and its target sequences (SBSs) to specifically investigate three-dimensional chromatin structure upon Th2 activation, which is transcriptionally competent in *Il4, Il5* and *Il13* expression. Active and silent gene expression states can be characterized by certain epigenetic marks, such as DNA methylation, histone acetylation and histone methylation as well as accessibility to nuclease and transcription factors[1,2]. Here we examined active versus silent chromatin states at the level of higher-order structure. To this end, we compared chromatin looping events and *in vivo*-associated proteins in resting versus activated Th2 cells, covering a 200kb region that includes the previously studied-120kb region. Surprisingly, in unactivated Th2 cells, the 200kb cytokine region has some features in common with the transcriptionally-active structure, such as association with GATA3, STAT6, and Brg-1. The silent chromatin was also associated with acetylated histone H3 at K9/14, normally a mark for active chromatin, albeit at much lower levels than in active chromatin. Furthermore, silent chromatin contained the characterization of the 'poised state' in which the LCR at the 3' of *Rad50* was brought into close proximity with the cytokine gene promoters similar to the active chromatin. Nevertheless, these features cannot induce cytokine gene expression until SATB1 is expressed and allows formation of a novel, higher-order transcriptionally active chromatin structure that contains RNA polymerase II and c-Maf.

Our findings reveal the key roles of the nuclear architectural protein SATB1 in both the formation of transcriptionally active chromatin at the cytokine gene locus and the induction of cytokine gene transcription. Previous studies from our group have shown that SATB1 tethers its target genomic sequences onto the SATB1 network to regulate regional histone modifications and expression of genes[17,18]. In contrast to a model in which a single or only few SATB1 binding sites exist at each gene locus, we have shown that SATB1 is able to tether multiple sites in a gene cluster, and fold chromatin into many small loops. We observed that in activated cells, the multiple chromatin loops that form at this locus commonly had SATB1 at their base. Furthermore, SATB1 not only binds to individual SBSs at the base of these loops, but it also binds indirectly to other sequences at the bases of loops, based on our results with the SATB1 ChIP-loop assay. This dense looping spatially connects individual SATB1-bound sequences, including those that are separated by 170 kb, with promoters and regulatory sequences of

15

multiple genes, converging individual loop bases into a common 'core base.' Using the cytokine gene region as a probe in fluorescence *in situ* hybridization (FISH) studies, we showed that in activated Th2 cells (supplementary Fig. 3), the densely-looped structure forms *in cis,* and not *in trans,* between the homologous sister chromosomes. We propose that this transcriptionally active form of chromatin at the cytokine gene cluster in activated Th2 cells resembles a three-dimensional, ribbon-like structure fastened at the base by SATB1 (Fig. 9b). *Rad50* intronic sequences that are enriched in SBSs have important roles in folding chromatin. Having a core base where all far remote sequences are brought together would explain the numerous amplified bands detected in both 3C and SATB1 ChIP loop assays (Fig. 9a).

Using an RNAi strategy, we showed that SATB1 is indeed required for formation of this dense-loop configuration. When SATB1 expression was reduced by more than 90%, chromatin of D10 cells failed to form a dense loop structure upon activation with Con A. Rather, the chromatin retained the basic looping structure typically detected in unactivated cells. Consistent with the view that the three-dimensional chromatin structure is an important component of transcription, when SATB1 production was inhibited, expression of *Il4*, *Il5* and *Il13* was not induced in activated cells. SATB1 is required for the cytokine gene expression not only through the formation of dense chromatin loops in the 200kb region but also through induction of c-Maf, which is required for *Il4* expression. SATB1 is an upstream regulator for the c-Maf expression in Th2 cells. Therefore, SATB1 regulates the expression of the *Il4, Il5, Il13* genes in multiple ways. Most likely, induction of c-Maf upon activation is directly regulated by SATB1 by tethering BURs in the c-Maf locus onto the SATB1 protein network, similar to the induction of c-Myc in activated thymocytes[17].

In both resting and activated D10 cells, the 200kb region is bound by Th2-specific transcription factors, such as GATA3 and STAT6, which bind DNA at many loci. These loci are not restricted to promoter regions, with only a moderate local enrichment at some sites upon cell activation. Previously, it was shown that GATA3 and STAT6 are important for the intrachromosomal interactions observed in the 'poised' chromatin structure[10]. Our study shows that the structure formed by GATA3 and STAT6 is not sufficient for the formation of the transcriptionally competent structure. SATB1 acts at a different level from these Th2 specific factors and is required for chromatin to rapidly adopt a transcriptionally-active conformation in response to a stimulus. In resting cells, the Th2-specific transcription factors and the chromatin

remodeling factor Brg-1 are already associated with the 200kb region, and histone H3 is acetylated at K9/14, but not methylated at K9. These epigenetic features, however, are not sufficient to activate cytokine gene transcription. In addition to the formation of dense loops that are fastened by SATB1, transcriptionally active chromatin in cytokine region has additional features, including much elevated H3 acetylation at K9/14, an increased association with Brg-1, and most importantly, its association with RNA polymerase II and c-Maf. In light of SATB1's role in targeting chromatin modifying/remodeling factors to specific regions of chromatin[18], it is likely that tethering the cytokine gene locus onto the SATB1 network triggers the recruitment of RNA polymerase II, c-Maf, and Brg-1.

The urea-ChIP assay provides a 'snap shot' of what is happening in several million cells at a given time, rather than in individual cells. Therefore, it is formally possible that individual cells each have a different subset of looping events, and that an increased variety of looping events occur during cellular activation. Due to technical limitations, this alternative scenario cannot be experimentally excluded at this point. However, an increased variation in the loop events would not explain why efficiency of transcription would be favored. To better interpret the structure-function relationship for transcriptionally active chromatin, we propose that the advantage of folding transcriptionally-active chromatin that include far distal sequences into small dense loops would be to reduce the total physical volume occupied by the local three-dimensional architecture of chromatin, and thereby increase accessibility of specific chromatin regions to transcriptional regulatory factors. The dense, small-loop structure would promote the coordinate and efficient expression of multiple genes in a cluster by bringing distal regulatory sequences into close proximity. Even though small bundles of loops form within individual cells, there may also be a time-dependent interaction of factors with individual genomic sequences. A concept was previously proposed that stable transcriptional machinery is anchored to the nuclear skeleton, through which DNA revolves during transcription[61]. In this situation, RNA polymerase II and other factors anchored in transcriptional complexes could interact with different genomic sequences as DNA is continuously reeled into the core structure, which remains anchored to the SATB1 network. Alternatively, the RNA polymerase II/transcription factor complex might track along the DNA fiber. The third possibility is that RNA polymerase II and other regulatory factors have very fast on/off rates, as unexpectedly revealed for RNA polymerase I, glucocorticoid receptors and other factors, so their function could depend on

productive collision[62]. In any of these three scenarios, compacting genomic sequences into a small volume would allow for a more efficient, dynamic interaction between DNA sequences and transcriptional regulatory factors. We propose that SATB1 provides nuclear focal points that allow for the efficient assembly of the three-dimensional chromatin architecture for rapid transcriptional induction.

Materials and Methods

Cloning of SATB1-binding sequences

We cloned SATB1-binding sequences, *in vitro,* from the mouse cytokine gene cluster region using following procedures. We digested two Th2 BAC DNA clones {CT7-AAAIA8(LBNL M01) andCT7-219O10(LBNL M02)} with Sau3AI and incubated 10 μg BAC DNA, with 10 nM GST-SATB1 for 25min at room temperature at the presence of 10 ng/μl poly dT-dC, 2.5 μg/μl BSA in buffer C (10 mM HEPES, pH7.9, 50 mM KCl, 10% glycerol, 2.5 mM $MgCl_2$, 1 mM DTT). We incubated the above reaction with glutathione Sepharose 4B beads (Amersham Biosciences, Piscataway, NJ) for 20 min at room temperature and washed the DNA on beads four times with buffer C. We then incubated beads with elution buffer (20 mM glutathione, 50 mM Tris-HCl, pH8.0) for 10min at room temperature to elute the DNA from beads and subjected them to phenol-chloroform extraction before ethanol precipitation with glycogen. To clone *in vitro* SATB1-binding sequences from cytokine gene cluster locus, we performed ligation-mediated-PCR (LM-PCR) for the DNA at 20-25 cycles of PCR amplification. We cloned the PCR products to pBluescript and determined the sequences. We performed gel mobility shift assay to determine the *in vitro* binding ability between SATB1 and SATB1 binding sequences.

Culture of D10G4.1 cells

We obtained murine D10G4.1 conalbumin-specific type 2 helper T-cell line (D10), derived from the AKR./J mouse [63], from American Type Cell Collection (Manassas, VA), and maintained as described, with minor modifications[64] We stimulated D10G4.1 cells twice monthly with irradiated syngeneic splenocytes from I-A[k] and conalbumin or irradiated splenocytes from I-A[b]. Antigen presenting cells (APC) were added to D10G4.1 cell at a ratio of 10 to 1 and resuspended in RPMI-1640 supplemented with fetal calf serum (FCS) and 10U/ml recombinant interleukin (rIL)-2. We harvested cells 2 weeks after stimulation and 5 days after rIL2 supplement as

'resting' D10G4.1 cells. For D10G4.1 activation, we added 5 µg/ml ConA added to culture medium.

RNase protection assay

We prepared total RNA from resting D10G4.1 cells and activated D10G4.1 cells 6 hrs and 24 hrs after ConA (Sigma, St Louis, MO) treatment. We performed RNase protection assay using 10 µg total RNA per reaction with Riboquant Kit (BD Biosciences, San Jose, CA) according to the manufacturer's protocol. Mouse cytokine multi-probe template (BD Biosciences) supplemented with *Rad50* and *Kif3a* cDNA for antisense RNA were used to prepare ^{32}P-labeled RNA antisense probes using *in vitro* transcription kit (BD Pharmingen, San Jose, CA). We cloned *Rad50* and *Kif3a* cDNA fragments into PCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) using oligomers 5' cttggcttttctgggctgt -3' and 5'- gcacgtctgtttcttgagca -3' for *Rad50* and 5' taagaccgattcctccaacg -3' and 5'- tctttctgacccagccagat   -3' for *Kif3a*. The antisense riboprobes for *Rad50* and *Kif3a* generated by the *in vitro* transcription give the free probe length of 541bp and 711bp respectively, and contained 452 bases of *Rad50* sequence and 622 bases of *Kif3a* sequence. The ^{32}P-labeled RNA duplexes were analyzed by electrophoresis in 6% (w/v) polyacrylamide/8M urea gels.

Immunofluorescence staining

We fixed D10G4.1 cells with 4% paraformaldehyde and incubated the cells with rabbit anti-SATB1 antibody (1583)[14] and mouse monoclonal anti-GATA3 (Santa Cruz Biotechnology, Santa Cruz, CA) mouse monoclonal anti-RNA polymrase II (Abcam, Cambridge, MA), rabbit anti-c-Maf (Santa Cruz, CA), rabbit anti-STAT6 (Santa Cruz, CA), rabbit anti-Brg1 (Santa Cruz, CA), and subsequently incubated with Alexa-488 conjugated goat anti-mouse IgG and/or Alexa 594-conjugated goat anti-rabbit IgG (1:400) (Invitrogen). DNA was stained with DAPI (Sigma). We collected images by a DeltaVision microscope and processed with SoftWoRx (Applied Precision, Issaquah, WA).

Chromatin immunoprecipitation

To determine if any specific DNA sequences are bound to SATB1 *in vivo*, we performed urea-ChIP following the procedure previously described[16,51]. Briefly, we used formaldehyde to cross-

link chromatin in thymocytes, resting D10G4.1 and activated D10G4.1 cells. We then purified cross-linked genomic DNA using urea-gradient ultracentrifugation. After restriction digestion with *Sau*3AI, we performed immunoprecipitation of crosslinked chromatin with rabbit anti-SATB1 antibody (1583)[14], rabbit anti-acetyl-histone H3 (Lys9/14, Upstate, Charlottesville, VA), mouse anti-RNA polymerase II (Abcam, Cambridge, UK), rabbit anti-c-Maf, mouse anti-GATA3, rabbit anti-STAT6, rabbit anti-Brg1 (all from Santa Cruz Biotechnology), and using pre-immune rabbit serum as a control. We reversed the crosslinking of ChIP samples with 100 μg/ml RNaseA and 250 μg/ml proteinase K digestion overnight, followed by incubation at 68°C for 6 hrs. We quantified IP DNA and input DNA, after reversing crosslinking, using Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen). We performed real-time PCR on immunoprecipitated DNA (IP DNA) using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) on ABI 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA). We employed absolute quantification method to quantify target DNA fragments in IP DNA using the standard curve prepared from input DNA for each primer pair. We carried out real-time PCR in triplicate to sextuplicate at 50°C for 2 min, and 95°C for 2 min, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. We determined the ratio R, where R=(moles of target sequence in IP fraction/moles of total IP DNA)/(moles of target sequence in input DNA/moles of total input DNA). This measurement gives relative fold difference in quantity of IP DNA prepared from resting and activated D10 cells using input DNA as a common reference for each primer set as described[65].

3C Assay and ChIP-loop assay

We performed the chromatin conformation capture (3C) assay essentially as described[4], except that we used crosslinked chromatin purified with urea gradient ultracentrifugation, as previous described[51]. We combined chromatin immunoprecipitation and 3C assay to perform ChIP-loop assay[13] which including the following steps: 1. *In vivo* cross-linking. We incubated 1×10^8 D10G4.1 cells in RPMI-1640 culture medium that contained 1% buffered formaldehyde at 37°C for 10 min and then at 4°C for 2 h. After cross-linking, we washed the cells twice with ice-cold PBS, and then lysed cells in a solution of 4% SDS in 10 mM Tris, pH 8.0, 1 mM EDTA. 2. Urea gradient ultracentrifugation. We purified cross-linked DNA and proteins from uncross-linked free proteins by centrifugation at 30,000 rpm for 16 h in Beckman SW41 rotor through a gradient

of 5-8 M urea prepared in 10 mM Tris, pH 8.0, 1 mM EDTA. We collected cross-linked chromatin at the bottom of urea gradient and dialyzed it against 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5mM EGTA, 5% glycerol overnight. **3**. Immunoprecipitation: We digested 60 µg DNA of cross-linked chromatin with *Sau*3AI in 500 µl of reaction buffer at 37°C overnight. We added 1%NP40 to digested chromatin and pre-cleared the sample by incubation with 50 µl of a 50% suspension of protein A-Sepharose 4B beads (Sigma) for 1 h at 4°C with rotation. We removed beads by brief centrifugation, and repeated pre-clearing crosslinked chromatin by adding 10 µl of pre-immune serum and incubated at 4°C for 1 h, followed by incubation with 40 µl of protein A-Sepharose 4B beads at 4°C for 1 h with rotating. After centrifugation, we incubated pre-cleared chromatin with pre-immune rabbit serum, anti-SATB1 antibody at 4°C for 4hr, and then with protein A Sepharose 4B beads at 4°C overnight with rotation. We washed beads four times with 1.0% NP-40 in PBS and two times with washing buffer (10 mM Tris-HCl, pH 8.0, 0.25 M LiCl, 0.5% NP-40, 0.5% DOC, 1 mM EDTA). **4.** Ligation: We resuspended the beads with ChIP DNA in 50 µl ligation buffer and allowed DNA to be ligated overnight at 16°C using T4 DNA ligase. **5.** Reversal of crosslinking. We digested the samples sequentially with 100 µg/ml RNaseA and 250 µg/ml proteinase K, treated at 68 °C for 6 hrs to revert crosslink, and subjected to phenol/chloroform extraction prior to ethanol precipitation. **6**. PCR amplification: We designed two primers (forward and reverse) in each of Sau3A DNA fragments 2-19 and a forward primer in DNA fragment 1 and a reverse primer in DNA fragment 2. Using combination of forward and reverse primers from different DNA fragments, we amplified ligated DNA with AmpliTaq Gold polymerase (Applied Biosystems) using 1 cycle of 94°C for 9 min, 35-40 cycle of 94°C for 30 sec, 58°C for 40 sec and 72°C for 30sec, and 72°C for 5min. The purification of crosslinked chromatin through urea gradient centrifugation allows more quantitative digestion with Sau3A. Therefore, the pattern of ligation products remain unchanged even after an additional 10-20 cycles of PCR amplification. We confirmed all the ligation products by cloning the PCR products, followed by DNA sequencing of the insert.

To correct for differences in crosslinking, ligation, PCR amplification efficiencies or sample loading, we prepared a control template set with DNA fragments from cytokine gene cluster locus and from the β-actin locus. For preparing cytokine gene cluster locus control template, we digested a BAC clone DNA spanning over cytokine locus with *Sau3AI* and religated with T4 ligase at a DNA concentration of 100 ng/µl. All possible ligation products were

present in the sample (data not shown). For preparing β-actin locus control template, we used two closely located neighboring fragments to design primers in the β-actin locus (supplementary Fig. 1c). The *Sau3A*I fragments between primers S2 and S3 are separated by 686bp. Upon *Sau3AI* digestion and ligation, we expect to obtain a PCR product of 149bp. The PCR amplification reproducibly occurs owing to the short distance. To get DNA template including equimolar DNA from the cytokine gene cluster locus and β-actin locus for 3C control, we cloned DNA fragment from actin locus between S1 and S4 primers with TA cloning kit (Invitrogen). Then, we mixed equimolar actin control DNA with Th2 BAC control DNA, followed by digestion and re-ligation, We also added actin primers in D10G4.1 3C sample as an internal control. We labeled PCR products by including 0.1μl {α-^{32}P} dATP (10 mCi/ml) and 0.1μl {α-^{32}P} dCTP (10 mCi/ml) in each reaction and resolved with 6% polyacrylamide gel electrophoresis. We quantified PCR products with Storm phosphoimager and ImageQuant software (Amersham Biosciences, Piscataway, NY). For the 3C assay, we measured signal of each reaction from D10G4.1 3C samples and control templates(described above), and calculated relative crosslinking frequency using the formula illustrated in supplementary Fig.1c, which corrects for any differences in PCR amplification efficiencies, crosslinking and ligation efficiencies, the amounts of the templates. We used the equivalent amount of original DNA (1ng) used for 3C assay and IP DNA for ChIP-loop assay. For the ChIP-loop assay, relative crosslinking frequency was calculated in the similar manner as the 3C assay. However, instead of using S2 and S3 primers for β-actin locus to correct the amount of genomic DNA used for the 3C assay, we added the plasmid DNA containing the actin locus in the original IP-DNA to correct for the amount of the starting DNA template used.   All experiments performed for the 3C assay and ChIP-loop assay were repeated a minimum of three times and the data shown are the mean values of multiple experiments.   Standard errors were within 15% of the values shown in figures.

shRNA transfection

We transfected D10 cells with 2 μg PSM2c plasmids[66] containing the SATB1 shRNA (5'AATACAAGGCATTTGTTGGCAT 3' and 5'GCCAACAAATGCCTTGTATT3'), or non-silencing shRNA by electroporation using Nucleofector[TM] II Device (Amaxa). We selected stable transfected cells by growing D10 cells in the presence of 20 μg/ml puromycin (Sigma).

Western blotting

We prepared whole protein from D10 cells at 0 and 24 hrs after Con A treatment. We resolved protein on 10% SDS-PAGE and electrotransferred protein to Immobilon-P membrane (Millipore, Billerica, MA). We blocked the membrane with 1% gelatin in TBS solution plus 0.1% Tween 20 (TBST) overnight at 4°C. We probed the membrane with a rabbit anti-SATB1 polyclonal antibody (Dickinson et al, 2002) or Rabbit anti-γ-tubulin (Sigma) diluted 1:2000 in TBST for 1 hr, then, a goat anti-rabbit horseradish peroxidase (HRP)–conjugated antibody (Bio-Rad, Hercules, CA) diluted 1:10,000 in TBST for 1 hr at room temperature. We detected signal with the SuperSignal West Pico Chemiluminescenct Substrate (Pierce, Rockford, IL).

FIGURE LEGENDS

Figure 1. Upon D10 (Th2) cell activation, SATB1 expression is induced and cytokine expression levels increase. a. RNase protection assay was performed on total RNA isolated from D10 cells 0, 6 and 24 hr after Con A activation. Comparing expression levels of genes in the 200 kb cytokine gene cluster locus before and after cell activation, *Il4, Il13, and Il5* transcripts level increased dramatically upon cell activation, whereas *Rad50* and *Kif3a* expression levels remain low. b, Immunofluorescence staining of D10 cells using antibodies against SATB1 and GATA3. SATB1 expression cannot be detected in resting D10 cells (left, top panel), but is expressed in activated cells, where it forms a network (right, top panel). GATA3 is expressed in both resting D10 cells (left, middle panel) and activated D10 cells (right, middle panel). After activation, SATB1 and GATA3 mostly colocalize in D10 cells (bottom panel).

Figure 2. Chromatin immunoprecipitation (ChIP) analysis of the cytokine gene cluster in D10 (Th2) cells reveals SATB1 binding sequences (SBSs). Schematic representation of the cytokine gene cluster locus and the primer sets designed for ChIP analysis (top panel). PCR amplification results using the primer sets (bottom panel) for genomic DNA control samples (top row), for Sau3A-digested, cross-linked chromatin, purified through urea-gradient centrifugation and precipitated with anti-SATB1 antibodies, for resting and activated D10 cells, (middle two rows), and for Sau3A-digested cross-linked chromatin immunoprecipitated prepared from activated D10 cells using pre-immune serum as a control (bottom row). We used typically 25-28 cycles of PCR amplification for this study. The specific data shown was produced with 28 cycles. Up to 30 cycles, the specificity of the PCR signals remains unchanged.

Figure 3. Chromatin loop analyses from SBS-C1 and SBS-C9 reveal dense SATB1-bound chromatin looping upon D10 activation. SBS-C1 and SBS-C9, indicated by red stars on the map, interact with cytokine promoters, LCR and other regulatory elements in cytokine gene cluster of D10 cells depending on the resting or activation status. Schematic representation of the cytokine gene cluster locus and the positions of the twenty Sau3A DNA fragments selected for the ChIP assay are shown. Forward and reverse primers are designed within these DNA fragments. Black lines indicate the DNA fragments that are located in spatially close proximity in resting D10 cells. Pink lines indicate the DNA fragments that show much enhanced

24

interactions upon D10 cell activation.   Red lines indicate those positions that are newly brought to close proximity after activation.   In activated D10 cells, all DNA fragments interactions shown by black, pink and red lines are observed. **a**, Relative crosslinking frequencies between fragment 2 (SBS-C1) as a fixed reference point and the other fragments of the locus (histograms) and the ligation products by PCR (gels), as determined by 3C assay with resting D10 cells (top panel) and activated D10 cells (middle panel). The lower panels show the results of the ChIP-loop assay using SATB1 to immunoprecipitate Sau3A digested chromatin fragments from activated D10 cells. **b**, Relative crosslinking frequencies between fragment 20 (SBS-C9) as a fixed reference point and other fragments of the locus (histograms), and the resulting ligation products by PCR (gels), based on the 3C assay in resting D10 cells (top panel) and activated D10 cells (middle panel). The lower panels show the results of the ChIP-loop assay using SATB1 to immunoprecipitate Sau-3A digested chromatin from activated D10 cells.   In **a** and **b**, lane numbers correspond to DNA fragments used as shown in the map, and lane M represents size marker.   Relative crosslinking frequency was calculated as described in Materials and Methods. Genomic DNA from D10 cells subjected to 3C assay was amplified with primers pairs from different DNA fragments or S2 and S3 primers of the β-actin locus, and their PCR products are indicated as Th2 or Actin, resptively. As control used to correct for ligation and amplification efficiencies, a mixture of Sau3A-digested and ligated BAC DNA covering the 200kb region and the plasmid DNA containing the β-actin locus was subjected to PCR amplification with primers from DNA fragments in the 200kb region or with S2 and S3 primers for actin, and their PCR products are indicated as Th2* or Actin*, respectively. For ChIP-loop assay, IP DNA was pre-mixed with the plasmid DNA containing the β-actin locus to correct for the amount of DNA used in each PCR reaction , and the PCR products are indicated as Actin*.   Experiments were repeated three times and the standard errors for relative crosslinking frequencies are lower than 15% at all points.

Figure 4. *Rad50* intronic SBSs contribute to chromatin loop organization of cytokine gene cluster region in D10 cells. a-e, Relative crosslinking frequencies between fixed fragments SBS-C3 (*a*), SBS-C4 (*b*), SBS-C5 (*c*), SBS-C6 (*d*) and 3' end of LCR (*e*), indicated by red stars on the map and other fragments of the locus, calculated from 3C assay on resting D10 cells (top panel), 3C assay on activated D10 cells (middle panel) and SATB1 ChIP-loop assay on activated

25

D10 cells (bottom panel) as described in Materials and Methods. The fixed reference points are shown by red bars in the histograms. Lane numbers correspond to DNA fragments as shown in the map. Lanes marked M represent size marker. While all *Rad50* intronic SBSs are involved in chromatin looping induced by activation, interaction involving the position at 3' end of LCR remains unchanged after activation. Experiments were repeated three times and the standard errors for relative crosslinking frequencies are lower than 15% at all points.

Figure 5. Th2 cytokine gene promoters are juxtaposed upon D10 activation, but *Rad50* promoter is not involved. **a-d**, Relative crosslinking frequencies (histograms) between fixed fragments of the *Il5* promoter (*a*), *Rad50* promoter (*b*), *Il13* promoter (*c*) and *Il4* promoter (*d*) indicated by red stars on the map and other fragments of the locus calculated from 3C assay on resting D10 cells (top panel), 3C assay on activated D10 cells (middle panel) and SATB1 ChIP-loop assay on activated D10 cells (bottom panel), as described in Materials and Methods. Lane numbers correspond to DNA fragments as shown in the map. Lanes marked M represent size marker. Experiments were repeated three times and the standard errors for relative crosslinking frequencies are lower than 15% at all points.

Figure 6. Association of Th2-specific factors, RNA polymerase II, Brg1 and acetylated histone H3 with the cytokine region.
a-f, Real-time PCR results from the Sau-3A digested crosslinked chromatin, purified through urea-gradient centrifugation, and immunoprecipitated from either resting D10 (blue line) or activated D10 cells (red line) with the use of antibodies against GATA3 (*a*); STAT6 (*b*); c-Maf (*c*), RNA polymerase II (*d*); Brg-1 (*e*); acetylated histone H3 at K9/14 (*f*), the activated status and the resting status are shown separately). Th2-specific GATA3 and STAT6 are essentially pre-assembled with the cytokine region in resting cells. Upon activation, their association is increased at specific sites indicated (a, b). c-Maf which is induced upon activation is strongly associated across the 200kb cytokine region, not limited to the *Il4* promoter region. RNA polymerase II becomes associated with the cytokine regions mainly after activation. Brg-1 association across the 200kb was increased upon activation. The level of histone H3 acetylation at K9/14 was dramatically increased upon activation. However, prior to activation, histone H3 in the cytokine region is found already acetylated to some degree. The map of the locus and the

positions of 24 primer sets used for real-time PCR are shown (top panel).   Standard error bars are shown.

Figure 7.   SATB1 is required for *Il4, Il5, Il13* expression and induction of c-Maf upon Th2 cell activation.

a) Western blot analysis of parental D10 cells (normal), stably transfected with a control construct expressing non-specific shRNA or an shRNA against SATB1 at 0 and 24 hr after ConA activation. b) RNase protection analysis of cells described under a) before and after ConA treatment at 6 and 24 hrs.   c) Immunostaining of parental D10 cells and D10 cells stably transfected with shRNA against SATB1 at 0 and 24 h after Con A activation.   The cells were stained with DAPI (DNA) and anti-c-Maf antibody (green).

Figure 8. SATB1 is required for dense looping in transcriptionally active chromatin.

The ligation products detected by PCR, based on the 3C assay in D10 cells stably transfected with the shRNA against SATB1 24 hr after ConA activation is shown. a) SBS-C1 (DNA fragment 2) or b) SBS-C9 (DNA fragment 20) indicated by red stars on the map was used as fixed reference points in this 3C assay. Lane numbers correspond to DNA fragments shown in the map and lanes marked by M represents size marker. The top panel (D10) used crosslinked genomic DNA for PCR amplification using primers in each DNA fragments (Th2) and with S2 and S3 primers for internal β-actin locus (Actin) to normalize the DNA amount used. To normalize amplification efficiency and ligation efficiency, pre-mixed, Sau3A-digested and ligated BAC clones and a plasmid DNA construct containing the β-actin locus was amplified with above primers and their PCR products are indicated as Th2* and Actin* as described in Fig. 3 legend and Materials and Methods.

Figure 9.   Summary of activation-dependent looping events and a model of transcriptionally active chromatin.

a) Summary of 3C and ChIP-loop assays of the cytokine regions in resting and activated D10 cells. Black lines connecting two positions indicate juxtaposition of these sites in resting cells. Pink lines represents two positions that show much increased crosslinking frequencies after

activation. Red lines represents two positions that are newly brought into spatially close proximity upon activation (based on both 3C and ChIP-loop assays). b) A schematic diagram based on the looping events shown in a). All small loops are converged to a common core base bound to SATB1(blue balls). This is based on an assumption that all looping events can occur in a single cell. Direct SATB1-binding site are shown by black vertical arrowheads. The crosslinking frequency for each ligation product generated between any two positions corresponds to the peak of a parabola connecting the positions. Based on the urea-ChIP data shown in Fig. 2 where SATB1 binding *in vivo* to BURs was detected most readily and predominantly in the 200kb region under the condition employed, it is possible that at a given time point when chromatin was crosslinked, only a small subfraction of cells was captured forming the densely looped structure.

Supplementary Figure 1. ChIP loop assay and 3C assay to determine chromatin structure of cytokine gene cluster region. (a) Schematic presentation of the Th2 cytokine locus. We used Sau3AI restriction enzyme digested fragments for 3C assay. Primers designed near Sau3AI sites are shown and designated as 1 through 20. (b) Diagram for ChIP-loop assay and 3C assay. After formaldehyde crosslinking, we purified crosslinked chromatin by urea gradient ultracentrifugation to remove un-crosslinked protein and proceeded to 3C assay as described in Methods. For ChIP-loop assay, we performed chromatin immunoprecipitation before intra-molecular ligation to identify the chromatin loop defined by specific protein. (c) The genomic DNA prepared for either ChIP-loop or 3C assay as described above was subjected to PCR amplification using various combination of forward and reverse primer pairs derived from the DNA fragments (1-20). These PCR products derived from the 200kb cytokine locus using genomic DNA is designated Th2. We used the β-actin locus (with primers S2 and S3) as an internal control to normalize any difference in the amounts of the genomic DNA, crosslinking and ligation efficiencies. The *Sau3A*I fragments between primers S2 and S3 are separated by 686bp. Upon *Sau3AI* digestion and ligation, a PCR product of 149bp are generated owing to the short distance. The PCR products using S2 and S3 primers are indicated as Actin. To correct for ligation efficiency and amplification efficiency of different primers, a mixture of plasmid DNA containing the β-actin locus between S1 and S4 primers and two BAC clones covering the 200kb cytokine region (see Materials and Methods) was subjected to PCR amplification with the same

series of primer pair combination from different DNA fragments. The PCR products derived from the BAC clones and Actin plasmid DNA are indicated by Th2* and Actin*, respectively. We calculated relative crosslink frequency using the formula shown, which corrects for any differences in PCR amplification efficiencies, crosslinking and ligation efficiencies, the amounts of the template initially used and the size of the PCR products.

Supplementary Figure 2. Immunolocalization of RNA polymerase-II, c-Maf, STAT6, Brg1 and SATB1 in resting and activated D10 cells.

The immunostaining data show that RNA polymerase II was present in both resting and activated cells. However, similar to SATB1, c-Maf is induced only after activation.   The levels and cellular localization of STAT6 and Brg-1 appear similar before and after activation. All of these factors once expressed appear co-localized to a large extent in D10 nuclei.

Supplementary Fig.3. Fluorescence *in situ* hybridization (FISH) analysis for the 200kb cytokine region.

The two BAC clones covering the 200kb cytokine region were used as FISH probes on activated D10 cells,   following the method previously described[17].   The results obtained from the experiment show that there is no interchromosomal interactions for the cytokine gene region which we studied.

REFERENCES

1.   Jenuwein, T. & Allis, C.D. Translating the histone code. *Science* **293**, 1074-80. (2001).
2.   Wilson, C.B., Makar, K.W., Shnyreva, M. & Fitzpatrick, D.R. DNA methylation and the expanding epigenetics of T cell lineage commitment. *Semin Immunol* **17**, 105-19 (2005).
3.   Klenova, E. & Ohlsson, R. Poly(ADP-ribosyl)ation and epigenetics. Is CTCF PARt of the plot? *Cell Cycle* **4**, 96-101 (2005).
4.   Dekker, J., Rippe, K., Dekker, M. & Kleckner, N. Capturing chromosome conformation. *Science* **295**, 1306-11 (2002).
5.   Tolhuis, B., Palstra, R.J., Splinter, E., Grosveld, F. & de Laat, W. Looping and interaction between hypersensitive sites in the active beta-globin locus. *Mol Cell* **10**, 1453-65 (2002).
6.   Palstra, R.J. et al. The beta-globin nuclear compartment in development and erythroid differentiation. *Nat Genet* **35**, 190-4 (2003).
7.   Drissen, R. et al. The active spatial organization of the beta-globin locus requires the transcription factor EKLF. *Genes Dev* **18**, 2485-90 (2004).
8.   Vakoc, C.R. et al. Proximity among distant regulatory elements at the beta-globin locus requires GATA-1 and FOG-1. *Mol Cell* **17**, 453-62 (2005).
9.   Murrell, A., Heeson, S. & Reik, W. Interaction between differentially methylated regions partitions the imprinted genes Igf2 and H19 into parent-specific chromatin loops. *Nat Genet* **36**, 889-93 (2004).
10.   Spilianakis, C.G. & Flavell, R.A. Long-range intrachromosomal interactions in the T helper type 2 cytokine locus. *Nat Immunol* **5**, 1017-27 (2004).
11.   Spilianakis, C.G., Lalioti, M.D., Town, T., Lee, G.R. & Flavell, R.A. Interchromosomal associations between alternatively expressed loci. *Nature* **435**, 637-45 (2005).
12.   Liu, Z. & Garrard, W.T. Long-range interactions between three transcriptional enhancers, active Vkappa gene promoters, and a 3' boundary sequence spanning 46 kilobases. *Mol Cell Biol* **25**, 3220-31 (2005).
13.   Horike, S., Cai, S., Miyano, M., Cheng, J.F. & Kohwi-Shigematsu, T. Loss of silent-chromatin looping and impaired imprinting of DLX5 in Rett syndrome. *Nat Genet* **37**, 31-40 (2005).
14.   Dickinson, L.A., Joh, T., Kohwi, Y. & Kohwi-Shigematsu, T. A tissue-specific MAR/SAR DNA-binding protein with unusual binding site recognition. *Cell* **70**, 631-45. (1992).
15.   Alvarez, J.D. et al. The MAR-binding protein SATB1 orchestrates temporal and spatial expression of multiple genes during T-cell development. *Genes Dev* **14**, 521-35. (2000).
16.   de Belle, I., Cai, S. & Kohwi-Shigematsu, T. The genomic sequences bound to special AT-rich sequence-binding protein 1 (SATB1) in vivo in Jurkat T cells are tightly associated with the nuclear matrix at the bases of the chromatin loops. *J Cell Biol* **141**, 335-48. (1998).
17.   Cai, S., Han, H.J. & Kohwi-Shigematsu, T. Tissue-specific nuclear architecture and gene expression regulated by SATB1. *Nat Genet* **34**, 42-51 (2003).
18.   Yasui, D., Miyano, M., Cai, S., Varga-Weisz, P. & Kohwi-Shigematsu, T. SATB1 targets chromatin remodelling to regulate genes over long distances. *Nature* **419**, 641-5. (2002).
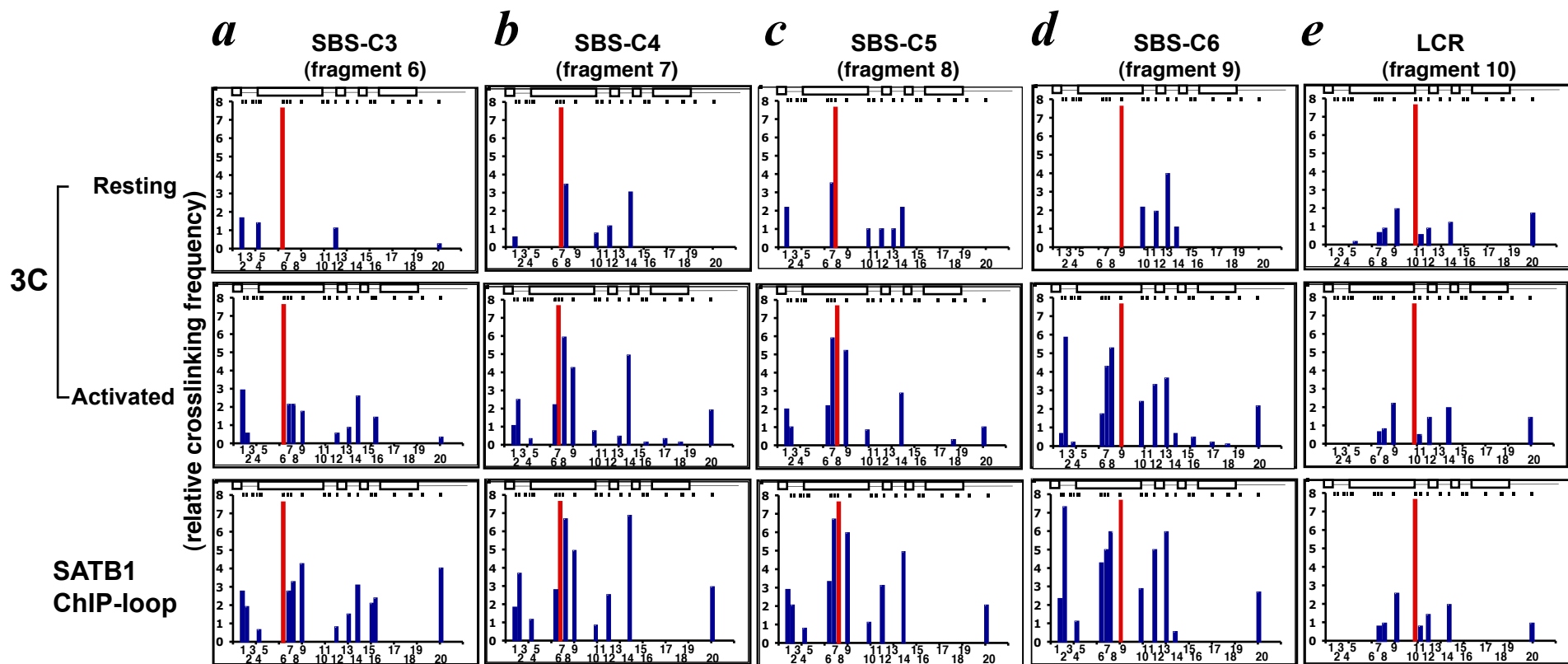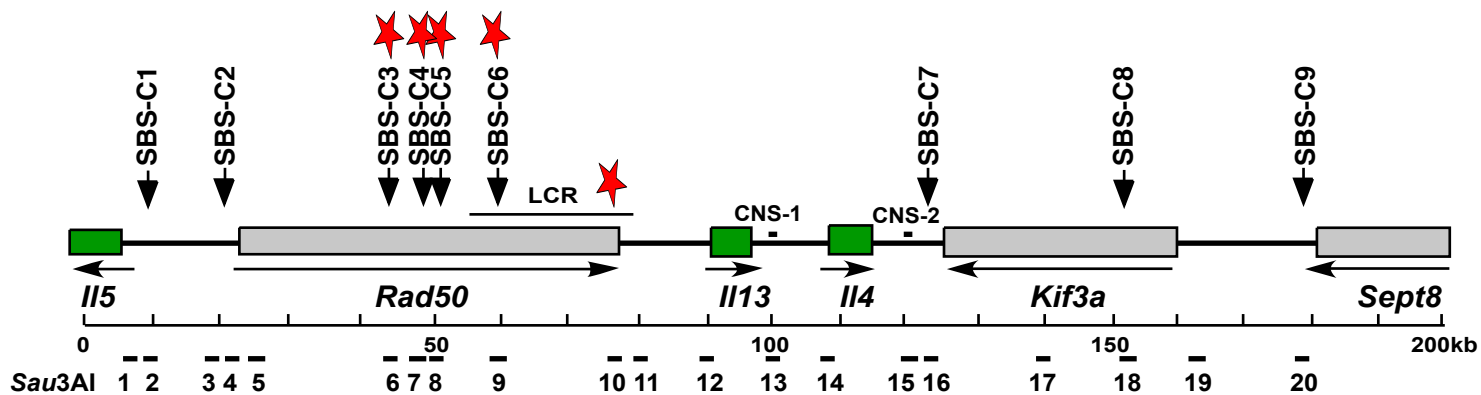
19.   Kohwi-Shigematsu, T. & Kohwi, Y. Torsional stress stabilizes extended base unpairing in suppressor sites flanking immunoglobulin heavy chain enhancer. *Biochemistry* **29**, 9551-60. (1990).

20.   Wen, J. et al. SATB1 family protein expressed during early erythroid differentiation modifies globin gene expression. *Blood* **105**, 3330-9 (2005).

21.   Hawkins, S.M., Kohwi-Shigematsu, T. & Skalnik, D.G. The Matrix Attachment Region-binding Protein SATB1 Interacts with Multiple Elements within the gp91phox Promoter and Is Down-regulated during Myeloid Differentiation. *J Biol Chem* **276**, 44472-44480. (2001).

22.   Dobreva, G., Dambacher, J. & Grosschedl, R. SUMO modification of a novel MAR-binding protein, SATB2, modulates immunoglobulin mu gene expression. *Genes Dev* **17**, 3048-61. (2003).

23.   FitzPatrick, D.R. et al. Identification of SATB2 as the cleft palate gene on 2q32-q33. *Hum Mol Genet* **12**, 2491-501. Epub 2003 Jul 29. (2003).

24.   Britanova, O., Akopov, S., Lukyanov, S., Gruss, P. & Tarabykin, V. Novel transcription factor Satb2 interacts with matrix attachment region DNA elements in a tissue-specific manner and demonstrates cell-type-dependent expression in the developing mouse CNS. *Eur J Neurosci* **21**, 658-68 (2005).

25.   Pavan Kumar, P. et al. Phosphorylation of SATB1, a global gene regulator, acts as a molecular switch regulating its transcriptional activity in vivo. *Mol Cell* **22**, 231-43 (2006).

26.   Dobreva, G., Dambacher, J. & Grosschedl, R. SUMO modification of a novel MAR-binding protein, SATB2, modulates immunoglobulin mu gene expression. *Genes Dev* **17**, 3048-61 (2003).

27.   Jones, P.L. et al. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat Genet* **19**, 187-91 (1998).

28.   Nan, X. et al. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* **393**, 386-9. (1998).

29.   Amir, R.E. et al. Rett syndrome is caused by mutations in X-linked *MECP2*, encoding methyl-CpG-binding protein 2. *Nature Genetics* **23**, 185-188 (1999).

30.   Smale, S.T. & Fisher, A.G. Chromatin structure and gene regulation in the immune system. *Annu Rev Immunol* **20**, 427-62 (2002).

31.   Glimcher, L.H. & Murphy, K.M. Lineage commitment in the immune system: the T helper lymphocyte grows up. *Genes Dev* **14**, 1693-711 (2000).

32.   Ansel, K.M., Lee, D.U. & Rao, A. An epigenetic view of helper T cell differentiation. *Nat Immunol* **4**, 616-23 (2003).

33.   Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A. & Coffman, R.L. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* **136**, 2348-57 (1986).

34.   Paul, W.E. & Seder, R.A. Lymphocyte responses and cytokines. *Cell* **76**, 241-51 (1994).

35.   Seder, R.A. & Paul, W.E. Acquisition of lymphokine-producing phenotype by CD4+ T cells. *Annu Rev Immunol* **12**, 635-73 (1994).

36.   Fields, P.E., Kim, S.T. & Flavell, R.A. Cutting edge: changes in histone acetylation at the IL-4 and IFN-gamma loci accompany Th1/Th2 differentiation. *J Immunol* **169**, 647-50 (2002).
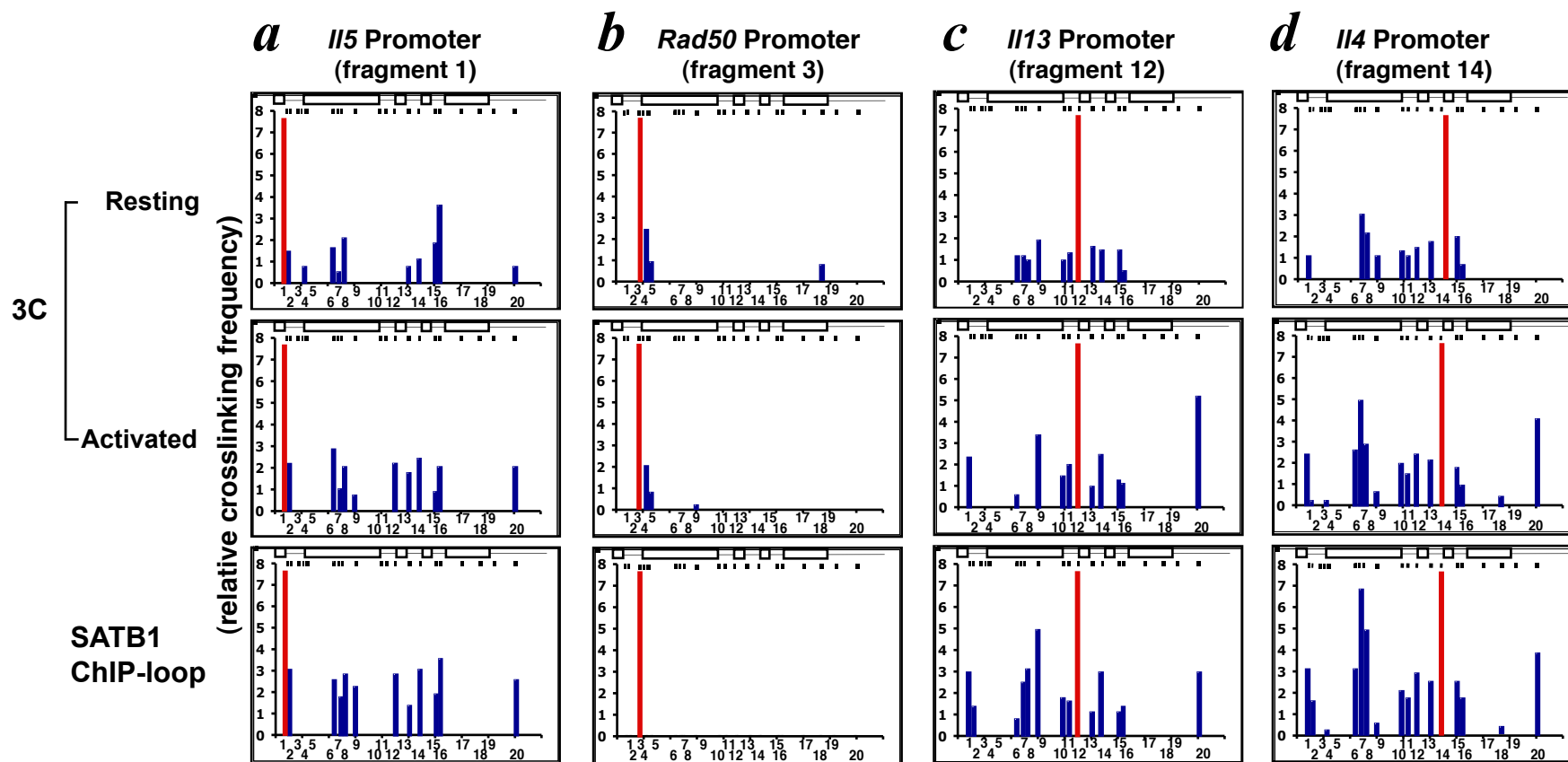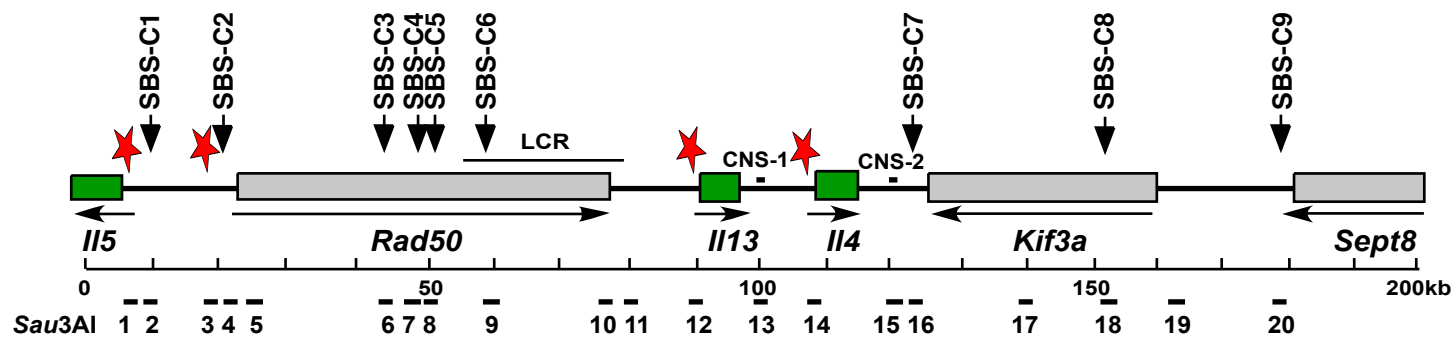
37.   Avni, O. et al. T(H) cell differentiation is accompanied by dynamic changes in histone acetylation of cytokine genes. *Nat Immunol* **3**, 643-51 (2002).

38.   Messi, M. et al. Memory and flexibility of cytokine gene expression as separable properties of human T(H)1 and T(H)2 lymphocytes. *Nat Immunol* **4**, 78-86 (2003).

39.   Guo, L. et al. In TH2 cells the Il4 gene has a series of accessibility states associated with distinctive probabilities of IL-4 production. *Proc Natl Acad Sci U S A* **99**, 10623-8 (2002).

40.   Lee, D.U., Agarwal, S. & Rao, A. Th2 lineage commitment and efficient IL-4 production involves extended demethylation of the IL-4 gene. *Immunity* **16**, 649-60 (2002).

41.   Makar, K.W. et al. Active recruitment of DNA methyltransferases regulates interleukin 4 in thymocytes and T cells. *Nat Immunol* **4**, 1183-90 (2003).

42.   Agarwal, S. & Rao, A. Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. *Immunity* **9**, 765-75 (1998).

43.   Agarwal, S., Avni, O. & Rao, A. Cell-type-restricted binding of the transcription factor NFAT to a distal IL-4 enhancer in vivo. *Immunity* **12**, 643-52 (2000).

44.   Takemoto, N. et al. Th2-specific DNase I-hypersensitive sites in the murine IL-13 and IL-4 intergenic region. *Int Immunol* **10**, 1981-5 (1998).

45.   Lee, G.R., Fields, P.E., Griffin, T.J. & Flavell, R.A. Regulation of the Th2 cytokine locus by a locus control region. *Immunity* **19**, 145-53 (2003).

46.   Fields, P.E., Lee, G.R., Kim, S.T., Bartsevich, V.V. & Flavell, R.A. Th2-specific chromatin remodeling and enhancer activity in the Th2 cytokine locus control region. *Immunity* **21**, 865-76 (2004).

47.   Grosveld, F., van Assendelft, G.B., Greaves, D.R. & Kollias, G. Position-independent, high-level expression of the human beta-globin gene in transgenic mice. *Cell* **51**, 975-85 (1987).

48.   Bulger, M. & Groudine, M. Looping versus linking: toward a model for long-distance gene activation. *Genes Dev* **13**, 2465-77 (1999).

49.   Festenstein, R. & Kioussis, D. Locus control regions and epigenetic chromatin modifiers. *Curr Opin Genet Dev* **10**, 199-203 (2000).

50.   Lee, G.R., Spilianakis, C.G. & Flavell, R.A. Hypersensitive site 7 of the TH2 locus control region is essential for expressing TH2 cytokine genes and for long-range intrachromosomal interactions. *Nat Immunol* **6**, 42-8 (2005).

51.   Kohwi-Shigematsu, T., deBelle, I., Dickinson, L.A., Galande, S. & Kohwi, Y. Identification of base-unpairing region-binding proteins and characterization of their in vivo binding sequences. *Methods Cell Biol* **53**, 323-54 (1998).

52.   Loots, G.G. et al. Identification of a coordinate regulator of interleukins 4, 13, and 5 by cross-species sequence comparisons. *Science* **288**, 136-40 (2000).

53.   Zhang, D.H., Cohn, L., Ray, P., Bottomly, K. & Ray, A. Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. *J Biol Chem* **272**, 21597-603 (1997).

54.   Zheng, W. & Flavell, R.A. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* **89**, 587-96 (1997).

55.   Zhu, J., Guo, L., Watson, C.J., Hu-Li, J. & Paul, W.E. Stat6 is necessary and sufficient for IL-4's role in Th2 differentiation and cell expansion. *J Immunol* **166**, 7276-81 (2001).
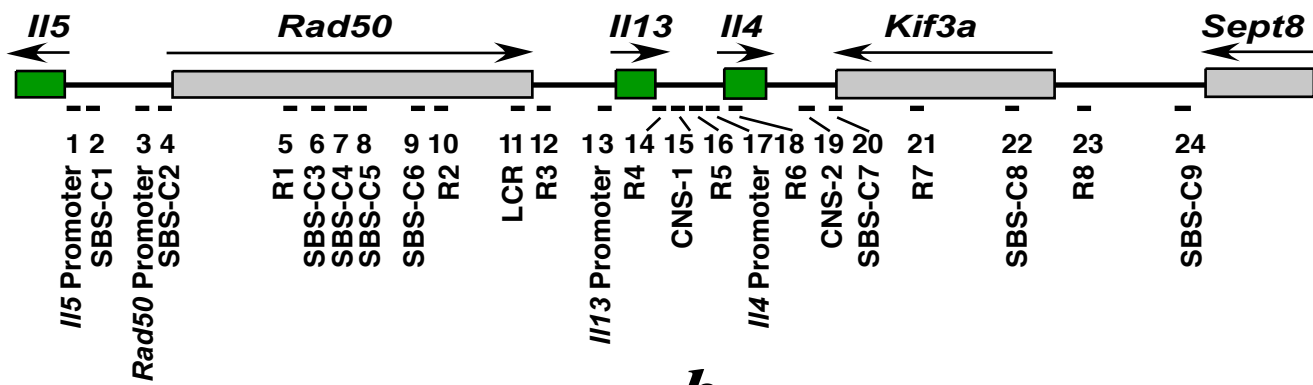
56.   Kurata, H., Lee, H.J., O'Garra, A. & Arai, N. Ectopic expression of activated Stat6 induces the expression of Th2-specific cytokines and transcription factors in developing Th1 cells. *Immunity* **11**, 677-88 (1999).

57.   Ho, I.C., Hodge, M.R., Rooney, J.W. & Glimcher, L.H. The proto-oncogene c-maf is responsible for tissue-specific expression of interleukin-4. *Cell* **85**, 973-83 (1996).

58.   Kim, J.I., Li, T., Ho, I.C., Grusby, M.J. & Glimcher, L.H. Requirement for the c-Maf transcription factor in crystallin gene regulation and lens development. *Proc Natl Acad Sci U S A* **96**, 3781-5 (1999).

59.   Hannon, G.J. RNA interference. *Nature* **418**, 244-51 (2002).

60.   Paddison, P.J., Caudy, A.A., Sachidanandam, R. & Hannon, G.J. Short hairpin activated gene silencing in mammalian cells. *Methods Mol Biol* **265**, 85-100 (2004).

61.   Cook, P.R. Predicting three-dimensional genome structure from transcriptional activity. *Nat Genet* **32**, 347-52 (2002).

62.   Hager, G.L. et al. Chromatin dynamics and the evolution of alternate promoter states. *Chromosome Res* **14**, 107-16 (2006).

63.   Kaye, J., Porcelli, S., Tite, J., Jones, B. & Janeway, C.A., Jr. Both a monoclonal antibody and antisera specific for determinants unique to individual cloned helper T cell lines can substitute for antigen and antigen-presenting cells in the activation of T cells. *J Exp Med* **158**, 836-56 (1983).

64.   Hopkins, S.J. & Humphreys, M. Simple, sensitive and specific bioassay of interleukin-1. *J Immunol Methods* **120**, 271-6 (1989).

65.   Litt, M.D., Simpson, M., Recillas-Targa, F., Prioleau, M.N. & Felsenfeld, G. Transitions in histone acetylation reveal boundaries of three separately regulated neighboring loci. *Embo J* **20**, 2224-35. (2001).

66.   Paddison, P.J. et al. Cloning of short hairpin RNAs for gene knockdown in mammalian cells. *Nat Methods* **1**, 163-7 (2004).
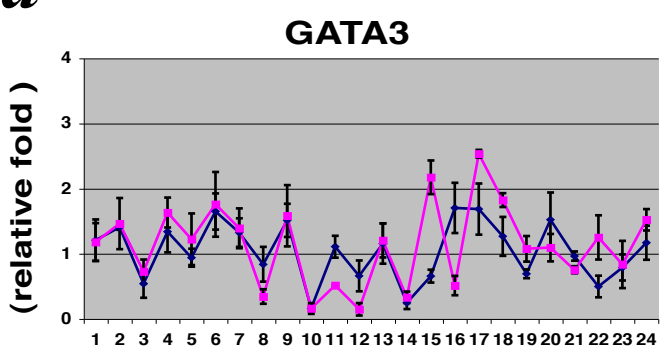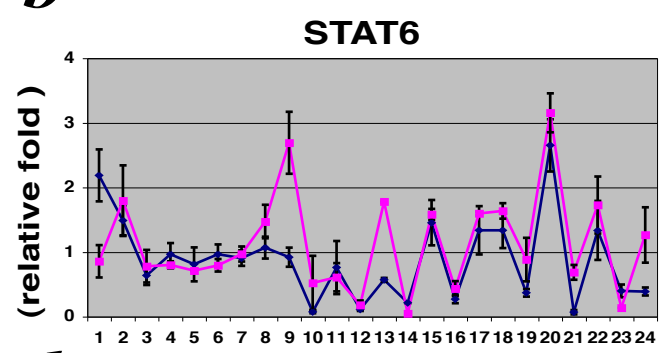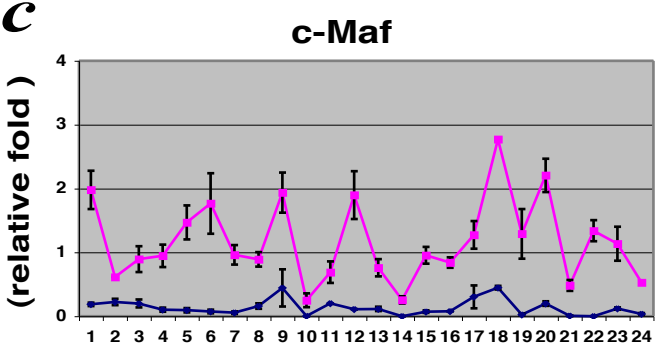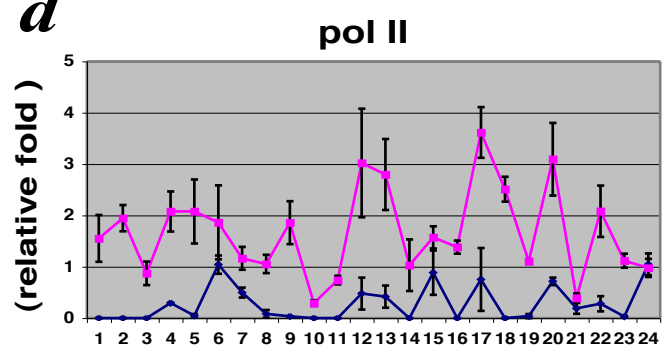
a

| 0 | 6 | 24 h | free probes |

Kif3a
Rad50
Il4
Il5
Il10
Il13
Il15
Il9
Il2
Il3
Ifnγ
I32
Gapdh

b

	Resting D10	Activated D10	
			SATB1
			GATA3
			Merged

a **SBS-C1 (fragment 2)**

b **SBS-C9 (fragment 20)**

3C
— Resting
— Activated

SATB1 ChIP-loop

(relative crosslinking frequency)

GATA3, STAT6, c-Maf, pol II, Brg1, H3 Lys9/14-Ac

a

Normal | Non-Silencing shRNA | SATB1 shRNA

0　24　0　24　0　24 h

SATB1

γTubulin

b

Normal | SATB1 shRNA | Non-Silencing shRNA

fp　0　6　24　0　6　24　0　6　24 h　fp

Kif3a
Rad50
Il4 ◄
IL5 ◄
Il10
Il13 ◄
Il15
Il9
Il2
Il3
Ifng
L32
Gapdh

c

Normal　SATB1 shRNA

0 h
c-Maf DAPI　c-Maf DAPI

24 h
c-Maf DAPI　c-Maf DAPI

SBS-C1 SBS-C2 SBS-C3 SBS-C4 SBS-C5 SBS-C6 LCR

CNS-1 CNS-2 SBS-C7 SBS-C8 SBS-C9

Il5 Rad50 Il13 Il4 Kif3a Sept8

0 50 100 150 200kb

Sau3AI 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

a SBS-C1

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Th2 — D10
Actin

Th2 — Control
Actin

b SBS-C9

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 M

Th2 — D10
Actin

Th2 — Control
Actin

a

b