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SATB1 Binding to an Element within the *Cd8α* Gene Enhancer Silences Expression of CD8αα$^+$ Intraepithelial Lymphocytes and Dendritic Cells

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Abstract

Special AT-rich Binding Protein 1 (SATB1) functions as a genome organizer capable of modulating chromatin architecture *via* binding to base-unpaired regions in chromatin. Previous transgenic studies identified a cis-acting element (termed L2a) that resides ~4.5 kb upstream of the mouse *Cd8α* gene within the Cluster 2 (CII) enhancer. L2a was shown to function as a CD8 silencer positively regulated by SATB1. To further investigate the function of L2a, we generated knock-in (KI) mice in which L2a sequences required for SATB1 binding were either mutated or completely abolished. Unexpectedly, based on the transgenic studies, L2a knock-in mice showed no observable defects in T cell development and only modest, variegated CD8αβ derepression in thymocytes, peripheral T cell subsets or Intraepithelial Lymphocytes (IELs). However, strong derepression of CD3-stimulated CD8αα+ IEL and CD8αα+ dendritic cells was observed in the guts of both knock-in strains. These observations suggest that SATB1-L2a interaction contributes primarily to silencing CD8αα expression.

Keywords: Genome; Intraepithelial lymphocytes; Transcription factors; Thymocytes

Introduction

A number of cis-regulatory elements and transcription factors (TFs) involved in control of the CD8αβ co-receptor have been identified over the past twenty years. These studies were fundamental in providing insight into regulation of CD8 expression, as well as how CD4 *vs.* CD8 fate choice is regulated in single positive (SP) and double positive (DP) thymocytes [1-4].

CD8 is typically expressed from closely linked *Cd8α* and *Cd8β* genes as a heterodimer on conventional T cells [5]. There are at least 5 cis-regulatory elements that achieve this complex regulatory and developmental stage, subset, and lineage-specific control [6-11] (Figure 1A). Several of these enhancers also have been ascribed to CD8αβ epigenetic regulation [12-15]. Further complicating the issue, activated CD8$^+$ and naïve CD8$^+$ T cells appear to regulate Cd8αβ differentially [16]. E8I, the first enhancer discovered within the Cd8 locus, was shown to be essential for expression of mature CD8$^+$ SP T cells and for CD8αα$^+$ intraepithelial lymphocytes (IEL) in the gut [8,9]. Knockout of E8I further established its requirement in transcription of CD8αα$^+$ γδTCR IEL and confirmed that conventional CD8αβ expression was unaffected [6,10].

A second, more recently identified enhancer, termed E8VI (Figure 1B), was shown to direct expression of CD8αα DC but not CD8αα IELs [17]. Thus, the cis-acting regulatory networks controlling CD8 expression continue to expand. Numerous TFs have been identified as critical regulators of Cd8, including T-BET, BLIMP-1, ID2, IRF4, BATF, ZEB2, TCF-1, EOMES, ID3, E proteins, BCL-6, FOXO1, MAZR, RUNX-3, BCL11B, and ThPOK [18,19].

Another implicated regulator, and the topic of this report, is Special AT-rich Binding protein 1 (SATB1). SATB1 functions in CD8 T cell genome organization [19,20] by forming complexes with chromatin modulators such as SWI/SNF [21-23]. Expression of SATB1 is established in hematopoietic stem cells and then restricted to T cell lineages [24,25].

SATB1 regulates numerous target genes involved in various cellular functions, including lymphoid lineage specification and/or commitment [24]. In SATB1 null mice, T cell development is interrupted at CD8αβ SP and DP stages [23]. SATB1 also is required for regulatory T (Treg) expression and function [26].

Transgenic studies from our laboratory established that SATB1 also functions as a silencer of CD8α transcription [27]. We identified a strong SATB1 binding site (termed L2a) near the 5' end of the E8III enhancer proximal to the second DNase Hypersensitivity (DH site) of cluster II (CII-2) (Figure 1).

In mice containing an L2a wild type (WT) transgene, CD8 reporter expression was silenced in both DP and CD8 SP thymocytes. When L2a WT transgenic mice were crossed onto a SATB1-deficient background, a fraction of variegated-expressing CD8SP thymocytes and splenocytes were significantly reduced.

The implication of these results-that SATB1 might overcome L2a silencing to re-express CD8 at a certain developmental stage(s)-was consistent with our finding that SATB1 is indispensable for re-initiation of CD8 transcription during the co-receptor reversal process of transition from CD4+CD8 low to CD8hiSP during positive selection [28].

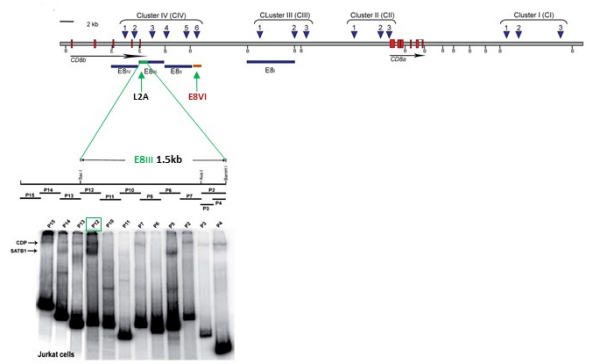


Figure 1 A. Map of the mouse *Cd8a* and *Cd8* gene loci. Horizontal arrows indicate the transcriptional orientation of the ~34 kb Cd8α and Cd8 1 locus (upper left, 2 kb scale bar) with exons denoted in red. DNaseI-hypersensitivity (DH) clusters I to IV (CI-CIV, indicated at the top) include individual DH sites (triangles). The horizontal blue bars denote established enhancers (E8I, E8II, E8III, and E8IV). The green boxes and arrows denote the recently discovered E8VI enhancer Sakaguchi et al (17) and L2a (described within) CD8αα enhancers. E, EcoR1; B. BamH1 restriction sites. Adapted from Sakaguchi, et al (17) and Yao, et al (27).**B.** Confirmation of SATB1 and CUX1 binding to L2a. Electrophoretic Mobility Shift Assays (EMSAs) were performed with Jurkat cell nuclear extract and 32P-radiolabeled E8III probes (p) spanning a 2.2 kb region that includes the ~200 bp L2a enhancer. The order of probes loaded in wells is not the same as that shown in schematic map above. The arrows indicate SATB1 or CDP/Cux complexes.

To further investigate the function of SATB1 as a CD8 repressor, we produced mutant knock-in mice either lacking or carrying mutations within the SATB1-binding L2a site. We observed no change in T cell development and only modestly increased expression of CD8 in thymocytes or peripheral T cell subsets. However, intestinal IELs from mutants lacking or mutated within L2a showed significant increases in CD8αα cells. Our results indicate that SATB1 binding near the 5' end of the E8III region selectively represses CD8αα expression predominantly within IELs.

Materials and Methods

Generation of L2a knock-in mice

The knock-in constructs (30 μg) were linearized with Not I and transfected into 129SvEV embryonic stem cells using electroporation. Transfected ES cells were cultured on irradiated SNL76/7 cells, and G418 (Genticin, GIBCO) was added (200 μg/ml) after one day. Ganciclovir was added (2 μM) after an additional two days, and individual ES colonies were isolated approximately nine days after transfection. Half of each colony was frozen, and the remainder was used to prepare DNA for identification of recombinants. Southern hybridization was used to screen for recombined positive clones using probe 1 or 2 for left arm or right arm respectively. Correctly targeted ES cell clones were injected into day 3.5 C57BL/6J blastocysts and transferred into CD1 pseudopregnant females. Male chimeric mice were backcrossed to C57BL/6 females, and agouti progeny were screened for germline transmission of targeted gene by Southern blot of tail DNA. Mice carrying targeted gene were crossed with EIIA ubiquitous Cre mice to delete the neo gene. After the removal of the neo cassette, PCR was used to determine the neo-deleted allele.

Preparation of genomic DNA from mouse tails

Mouse tails were digested in 300 μl of tail buffer (50 mM Tris (pH 8.0), 100 mM EDTA, 1% SDS, and 0.15 mg/ml Proteinase K) at 55°C overnight. Tails samples were extracted sequentially with 300 μl of phenol, phenol-chloroform (1:1), and chloroform, and the DNA was precipitated with 100 μl of 30% PEG and 1.5 M NaCl solution. DNA was pelleted (14,000 rpm, 15 min), washed once with 70% ethanol, dried, and resuspended in 100 μl of TE (10 mM Tris-HCl, (pH8.0), 1 mM EDTA).

Southern blot analysis

The purified genomic DNA (25 μg) was digested with restriction enzyme to completion. A 0.7% agarose gel was used to separate digested DNA by electrophoresis. The DNA was transferred to a nylon membrane (MSI), and the blot was hybridized overnight with random-primer labeled probes in Ultrahyb solution (Ambion). After hybridization, blot was washed twice with solution containing 2X SSC, 0.1% SDS at 55 °C for 5 min, then washed twice with 0.1X SSC, 0.1% SDS solution at 55 °C for 15 min. Blots were air dried briefly and exposed using a phosphoimaging cassette. The [32P] labeled probes were generated by random primer synthesis using a decaprime DNA labeling kit (Ambion).

Isolation of cells from mouse thymus, lymph nodes and spleen

Thymus, lymph nodes and spleen were removed from euthanized mice and placed into 60 mm dishes containing HBSS (Sigma) buffer. Tissues were passed through a 70 micron nylon cell strainer (BD Biosciences) to prepare single cell suspension. To remove red blood cells, isolated cells were incubated in RBC lysis buffer (0.5 M NH4Cl, 0.15 M Tris-HCl [pH 7.65]) for 5 min at room temperature. Cells were washed with HBSS and ready for desired treatment and analysis.

FACS staining

Isolated cells were washed with HBSS (Sigma) buffer twice at 1,000 rpm 4°C, and resuspended in Hanks buffer (HBSS with 2% FBS and 0.1% sodium azide) on ice. Cells were counted and 1×10^6 cells were used for subsequent staining. After incubation on ice with Fc-block (provided by Dr. Ellen Richie, M.D. Anderson Cancer Research Center) for 15 min, cells were stained with the desired antibodies for 45 min. Following two washes with 1 ml Hanks buffer, 1 ml HBSS once, cells were fixed using 1% paraformaldehyde and analyzed immediately. Cells requiring secondary antibody staining were incubated on ice with the appropriate reagent for 30 min after the wash steps of first staining. Cells were then washed and analyzed on a BD FACS calibur using CellQuest Pro software.

Cell sorting

Cells of interest were sorted and separated by a Magnetic Cell Sorting and Separation (MACS) System (Miltenyi Biotec GmbH). Briefly, cells were labeled by desired antibodies with magnetically labeled MicroBeads. After magnetic labeling, cells were passed through a separation column which was placed in a strong permanent magnet.

The magnetically labeled cells were retained in the column, while the unlabeled cells passed through. The retained fraction was eluted and used immediately for culture and subsequent studies.

Preparation of probes for EMSA

All the probes were end-labeled with [α-32P] dATPs using exo-Klenow enzyme (Biorad). L2a 200(L+S) probe and its mutant probes were cloned into pBluescript vector and excised out by appropriate restriction enzymes for end-labeling. EMSA probes for the DH cluster II fragment and E8III fragment were created by PCR. BamHI or EcoRI restriction sites were added to the 5' end of primers, and PCR amplified fragment were digested with both enzymes and purified for end-labeling (S-Figure 1).

Probe name	Primer Name	Sequence (5'-3')
P2	E3Bm3661F	TTA GGA TCC TAG GAC TCC CAA AGC
	E3BmR	CAC ACC TTT AAT CCC AGT GC
P3	E3Bm3661F	TTA GGA TCC TAG GAC TCC CAA AGC
	E3Er3844R	TCA GAA TTC AAG GTT CTC CAA CG
P4	E3Er3821F	AAT GAA TTC TAG CCG TTG GAG AAC
	E3BmR	CAC ACC TTT AAT CCC AGT GC
P5	E3Er3047F	TGA GAA TTC AGC AGC CAT CTT ACT CTC
	E3Bm3269R	TCT GGA TCC TGG GAT TAA AGG CAT GC
P6	E3Er3257F	TGT GGA TTC TTT AAT CCC AGC ACT CG
	E3Bm3464R	TAT GGA TCC TAG CTT GTC TGA GGT C
P7	E3Er3448F	AGT GAA TTC AGA CAA GCT AGG AGT G
	E3Bm3682R	TAT GGA TCC TGG CTT TGG GAG TCC TAG
P10	E3Er2840F	TAA GAA TTC CCA CAC CAC CAT GTA C
	E3Bm3071R	ACT GGA TCC TGA GAG AGT AAG ATG GCT G
P11	E3Er2685F	TCA GAA TTC TAG TGA GAG ACA GC
	E3Bm2867R	ATT GGA TCC AAG AAG AGT ACA TGG TG
P12	E3ScF	ACC ATC CTA ACA GAG CTC TC
	E3Bm2700R	TTA GGA TCC GCT GTC TCT CAC TAG
P13	E3Er2257F	TCA GAA TTC TAC TCA CTG AGA CAT C
	E3Bm2489R	ACT GGA TCC TGA GAG CTC TGT TAG
P14	E3Er2018F	TCA GAA TTC TGT CTT AGA GCA TCC TC
	E3Bm2280R	ACT GGA TCC AGT AGG ATG TCT CAG
P15	E3Er1682F	TCA GAA TTC TAC ACA GTC AGG AGA TC
	E3Er2030R	ACT GGA TCC TCT AAG ACA GAA GGT TG

S-Figure 1. Primers used to make EMSA probes for DH cluster II. All probes were end-labeled with [α-32P] dATPs using exo-Klenow enzyme. EMSA probes for the E8III region were created by PCR. BamHI or EcoRI restriction sites were added to the 5' end of primers, and PCR amplified fragment were digested with both enzymes and purified for end-labeling.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described by Dignam et al. [29]. All steps were performed at 4°C or on ice. Nuclear extracts (2-5 μg) were mixed with poly-(dI-dC,2 μg) in binding buffer (20 mM HEPES [pH 7.9], 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 10 mM DTT, and protease inhibitor cocktail). Binding reactions were performed in 25 μl total volume at room temperature for 5 min. After 20 min incubation with end-labeled probe (0.2 μg), samples were electrophoresed at 120 V for ~3 h through a 4% polyacrylamide gel (29:1) in 1X TBE buffer (90 mM Tris-HCl [pH 8.0], 90 mM boric acid, and 2 mM EDTA).

Gels were dried for 1 h and autoradiographed for 4 h, using a phosphoimage screen, or overnight using films with an intensifying screen at -80°C.

Transient transfection and luciferase assays in cultured cells

293T cell transfections were carried out using Fugene 6 (Roche) following the product instructions. L2a Firefly luciferase constructs were co-transfected with Renilla luciferase vectors into 293 cells. 36 h after transfection, cells were washed with PBS and resuspended in Lysis Buffer (Dual-Luciferase Reporter Assay System, Promega). Cell lysates were applied to dual-luciferase assay following product instruction. The Firefly intensities were normalized by Renilla intensities to obtain the relative activities.

In vitro T cell activation

Isolated splenocytes were washed and resuspended in RPMI culture media. Cells were distributed to 24-well plates at a concentration of 2.5 × 10^6/ml and 2 ml/well. Anti-CD3ε antibody (BD Biosciences) was added to the cells to a final concentration of 1 μg/ml. Cells were sampled at day 2 or day 4 and applied to FACS analysis.

Isolation of intestinal intraepithelial lymphocytes (IELs)

IELs were isolated by a modified method based on a procedure previously described [9].The small intestine was removed from euthanized mice and washed with RPMI medium. The small intestine was turned inside-out over a glass tubing and incubated in 30 ml of RPMI for 45 min at 37°C with low speed rotation to release the IELs. The released IELs were passed through a 70 μm cell strainer to filter out debris. Cells were centrifuged (1000 rpm, room temperature) and resuspended in appropriate volume of RPMI medium. Cells were then purified with Ficoll-Pague Plus (Amersham) centrifugation (2000 rpm, 30 min, room temperature), and washed with HBSS buffer.

L2a transgenic constructs and procedures

A human (h) CD2 reporter gene [30], containing the mouse CD4 exon I, a portion of intron I lacking the CD4 silencer, and the untranslated portion of exon II (a CD4 splicing module) was fused in frame to hCD2 cDNA appended by a SV40 polyadenylation site [31]. A PCR-amplified mouse Cd8α promoter [32] was inserted into the polylinker to make a construct termed Tg-α [9]. A wild type construct (L2aWT) was constructed by poly-linker insertion of a ~4 kb DH cluster II fragment, and an L2a deleted (L2aD) construct carries a deletion of the 210 bp L2a elements. Transgenic C57BL6 mice were created by pronuclear injection for each construct as previously detailed [27]. The number of integrated copies was determined by comparing signal intensities of wild type and transgene-containing bands in liver DNA by Southern blot analysis. We estimated that L2aWT and L2aD employed in this study carried 8 and 5 concatermized copies, respectively.

Results

Targeted deletion/knock-in approach to the function of L2a

We previously reported [27] that over-expression of transgenes carrying deletions of a 3.4 kb genomic region containing the L2a element (DH clusters CII-1 and CII-2; Figure 1A) led to aberrant thymocyte development. This was characterized by a large population of thymocytes that failed to extinguish *CD8* gene expression and concomitant decreases in thymic and peripheral CD8SP T cells. These results suggested that the L2a element, which is bound strongly by SATB1 and CDP/Cux (Figure 1B), acts to silence *CD8* gene

transcription. To further investigate the putative role of L2a in its natural *in vivo* chromosomal context, knock-in mice were produced with deletion of and mutations within L2a.

Generation of L2a knockout and knock-in mice

The targeting strategy is shown in Figure 2A and detailed further in Appendix I. We first generated a 200 bp deletion that included the SATB1 and CDP/CUX protected region and the L+S flanking region (termed KI- L2a; red box in Figure 2).

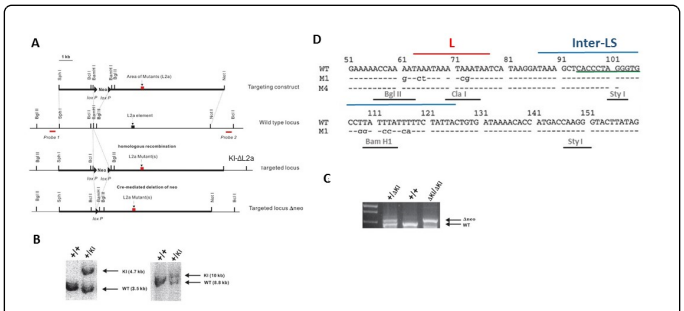


Figure 2. L2a knockout (KO) and knock-in (KI) strategies. **A.** Schematic of the targeting construct, the Cd8 locus before and after homologous recombination and the genomic locus after Cre recombinase-mediated deletion of the neo gene to create the 200 bp deletion of L2a (indicated in red). Restriction enzymes required for cloning and characterization are indicated with further details provided in Appendix 1. **B.** Southern blots of Bgl II (left) and Bcl I (right) digested DNA isolated from a wild-type (WT) ES cell clone (+/+) and from an ES cell clone after homologous recombination (+/KI); Left arm (Probe 1/ Bgl II) and right arm (Probe2/ Bcl I). **C.** PCR genotyping of the targeted locus after the deletion of the neo cassette. The neo cassette and loxP sites are flanked by primers that provide a 40 bp size difference for WT and Δneo bands. **D.** Knock-in mutants of SATB1 (M1) created by insertion into KI-ΔL2a. Shown are ~100 bp of L2a containing WT and mutated binding sites for SATB1. Amino acids are in single letter code; identities are indicated by dashes. KI-M1 was generated and cloned into the knock-in targeting construct (as indicated in A) to generate KI-M1 mice. M1 abolishes the binding of SATB1, but leaves the binding of CDP/Cux intact.

The wild type (WT) L2a 200 bp fragment was knocked back into the KI- L2a vector to create a sequence identical with the WT control (termed KI-WT; Figure 2). Comparative FACS analyses of KI-WT mice (which contained recombined loxP sites) in C57BL/6x129/Sv mice (from which ES cells were obtained) indicated no differences between expression of CD4, CD8 as other surface makers. We next created point mutations within amino acids required for SATB1 binding and then reinserted them into KI- L2a. This mutant termed KI-M1, carries 5 substitutions that abolish the binding of SATB1 to L2a, but leave the L2a binding site of CDP/CUX intact.

Expression of CD8 is unaltered in KI-M1 mice point-mutated within the SATB1 binding site within L2a

Cells were isolated from lymph nodes and thymus from homozygous KI-WT and KI-M1 mice, then stained with antibodies specific for CD4 and CD8α. FACS analyses of 5 independent founder strains (Figure 3) detected no alteration of CD8 expression. To examine whether loss of L2a resulted in T cell maturation defects, several developmental T cell surface markers were tested in CD4SP, CD8SP and DP thymocytes. KI-WT and KI-M1 mice showed similar expression of CD3, CD5, CD24, CD44 and CD69 molecules (Figure 3).

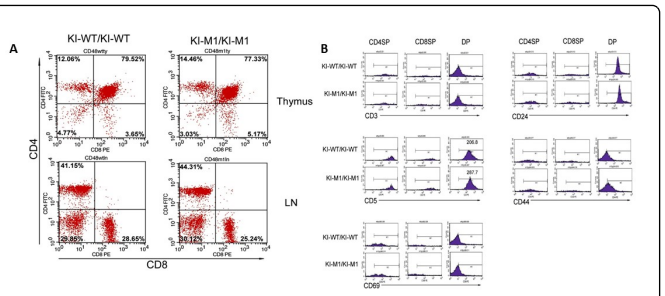


Figure 3. Loss of SATB1 binding to L2a has no effect on levels of CD8, CD4 or T cell markers in thymocytes or lymph nodes. A. Lymph node cells and thymocytes from KI-M1 and KI-WT controls were isolated from homozygous KI-WT and KI-M1 mice, stained with antibodies against CD4 and CD8α, and analyzed by flow cytometry. Percentages of each cell population are indicated. Data were representative of 5 independent embryonic stem (ES) cell founder strains. B. Expression of T cell surface markers is unperturbed in KI-M1 mice. The indicated developmental T cell surface markers were tested on CD4SP, CD8SP and DP thymocytes. KI-WT and KI-M1 mice showed similar expression levels of CD3, CD5, CD24, CD44 and CD69 as measured by MFI.

RT-PCR further confirmed that there were no changes in levels of CD8α (data not shown). These results indicated that the M1 mutation, which abolishes SATB1 binding, does not significantly perturb conventional CD8 expression nor T cell development.

Complete L2a elimination (KI- L2a) results in modest derepression of CD8 thymocyte expression

Four out of six independently derived KI- L2a mice showed only modest phenotypic changes in both thymic and LN-derived CD4 and CD8 T cell expression (Figure 4).

However, two additional founder lines demonstrated no apparent differences between KI and controls (data not shown). One explanation for the observed lack of correspondence is that loss of L2a results in a variegated phenotype. Variegated expression of the Cd8-Cd4 locus has been documented in several previous studies [12,27,33-35] and is readdressed in Discussion. In the mutation phenotype shown in Figure 4A, full L2a deletion resulted in a non-significant, upward trend in CD4SP (9.9% to 16.9%; $p \leq 0.07$), whereas CD8SP thymocytes were significantly increased (2.5%-4.0%, $p \leq 0.05$). No significant effect was observed in either CD4SP or in CD8SP lymph nodes (LN). However, analysis of thymocytes from KI- L2a mice for T cell developmental and activation markers (CD3, CD5 and CD69; Figure 4B,C) consistently detected a ~1.8-fold ($p \leq 0.05$) increase in thymic DP levels, consistent with a modest decline in TcR expression in L2a-deleted thymocytes.

Collectively the results indicated that germline deletion of the ~140 bp spanning the L2a element within the CII regulatory region (Figure 1A,1B) results in significant, but modest SATB1 derepression. These results were unanticipated based on the robust repression we observed

from this identical 140 bp segment in the transgene gain-of-function context [27].

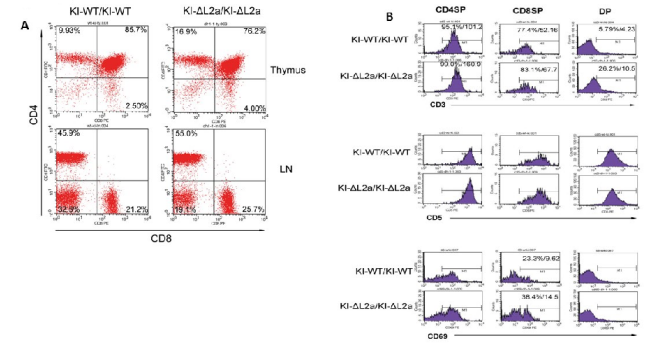


Figure 4. KI-ΔL2a mice show a modest increase in CD8SP expression in lymph nodes and thymus. A. Lymph node cells and thymocytes were isolated from homozygous KI-WT and KI- L2a mice, stained with antibodies against CD4 and CD8α and then analyzed by FACS. Percentages of each cell population are shown. The MFIs of CD4 and CD8 were similar in all populations. Three out of five KI- L2a homozygous mice had modest, statistically insignificant reduction in CD4 and modest statistical reduction ($p \leq 0.05$) in CD8 expression in thymocytes and lymph nodes. Results from one of the three homozygous KI- L2a mice are shown. B. Expression of T cell surface markers CD3, CD5 and CD69 are modestly altered in DP thymocytes from KI-WT mice. Changes in percentages and MFIs of positive cells are shown. Diagrammatic representation of the data of Figure 4B indicating that CD3 expression is significantly ($p \leq 0.05$) elevated on CD4CD8 DP KI-L2a thymocytes. For comparison, control levels of CD4SP, CD8SP and CD4CD8DP were adjusted to a value of 1 (dotted line). The data are averaged from 4 independent measurements with standard deviation indicated by vertical lines within each box.

CD8αα and CD8αβ expression is modestly increased in intraepithelial lymphocytes of KI and M1 mice

Intraepithelial lymphocytes (IELs) from gut and intestine express exclusively CD8αα homodimers [36,37]. L2a-M1 mice, in which SATB1 binding to L2a is eliminated by point mutation (Figure 2B), were tested for expression of CD8αα homodimer expression on their IELs.

TCRαβ+CD8β+, TCRαβ+CD8β- and TCRγδ+CD8β- IELs were isolated from mouse intestines, gated and stained for CD8α expression as described in Materials and Methods. Modestly increased CD8αα expression and MFI were observed on TCRαβ IEL derived from KI-M1 mice (Figure 5A). KI- L2a mice, in which ~1.5 kbp spanning L2a was deleted, also showed modest elevation of CD8αα homodimers on both TCRαβ+CD8β- and TCRγδ+ CD8β- IELs (Figure 5B).

These results showed a consistent trend ($p \leq 0.6$) towards elevated expression of CD8αα in IELs resulting from loss of SATB1 binding due to L2a mutation or deletion. The data suggest that SATB1 and L2a may act in concert to silence CD8αα expression in IELs.

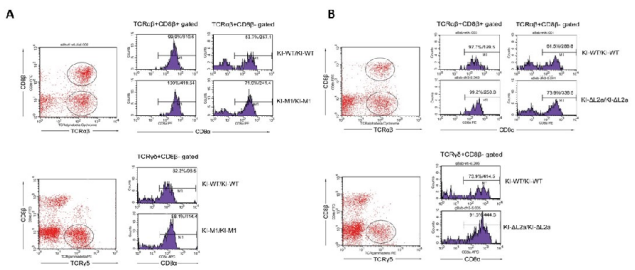


Figure 5. CD8αα is modestly increased on intraepithelial lymphocytes (IELs) of both KI-M1 and KI-ΔL2a mice. Expression of CD8αα homodimers in IELs was tested on KI-WT and KI-ΔL2a mice. IELs were stained, gated, and analyzed as described in the legend to Figure 4 and in Materials and Methods. Percentages and MFIs of positive cells are shown. A. KI-M1 point mutants within L2a show modest, statistically insignificant elevated expression of CD8αα homodimers on TCRαβ IEL. B. KI-ΔL2a mutant mice in which the 200 bp region spanning L2a is deleted show modest but statistically insignificant elevation of CD8αα homodimers on both TCRαβ+CD8β- and TCRγδ+CD8β- IELs.

The L2a element collaborates with E8I to promote robust CD8αα repression in activated IELs

It has been previously shown that CD8αα expression can be induced upon antigenic stimulation to promote the survival and differentiation of activated lymphocytes into memory CD8 T cells-a process controlled by the E8I enhancer [6].

The L2a element and DH cluster II region have been shown to collaborate with E8I in transgenic studies. Thus, we deemed it informative to determine whether L2a is involved in the induced expression of CD8αα in activated IELs.

Intestines from KI-WT, KI-M1 and KI- L2a homozygous mice were isolated, and following conversion to single cell lysates, were cultured and stimulated with anti-CD3ε antibody. CD8αα expression was analyzed by staining with thymic leukemia (TL) tetramers [38,39], shown previously to bind preferentially to the CD8αα homodimer [40-45].

Four days of culture led to ~8-fold activation in CD8αα expression in L2a-deleted KI-L2a mice ($p \leq 0.001$, Figure 6). Consistent with that result, L2a-point mutated KI-M1 showed ~10 fold stimulation in CD8αα expression as compared to KI-WT controls ($p \leq 0.001$).

These increases in CD8αα are consistent with the hypothesis that L2a is a silencer, and its deletion relieved repression of TCR-mediated (i.e., anti-CD3) induction of CD8αα expression-an event particularly critical to survival and differentiation of memory cells [46].

Splenic CD8αα$^+$ dendritic cell (DC) expression is modestly repressed by L2a

In the mouse spleen, a subset of dendritic cell also expresses CD8αα homodimers [47] which develop from both myeloid and lymphoid progenitors [48]. To test whether L2a contributes to dendritic cell expression, we employed a transgenic approach detailed previously [27] and summarized in Materials and Methods.

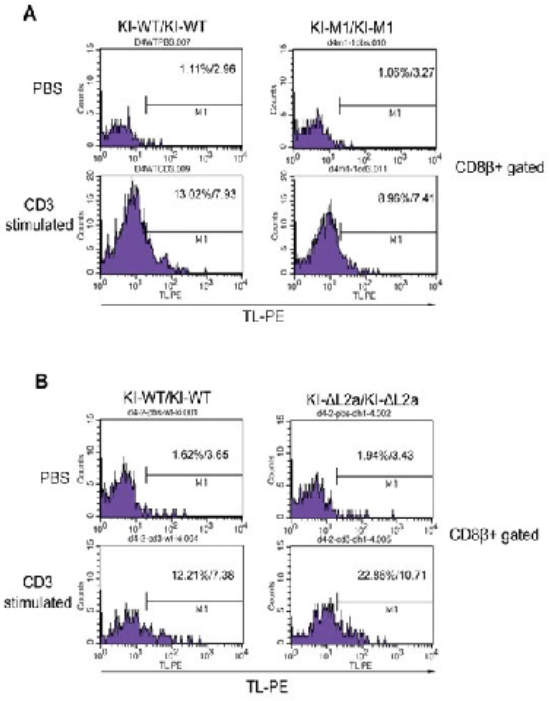


Figure 6. L2a collaborates with E8I for CD8αα expression in activated peripheral T cells. Splenocyte cultures from KI-WT, KI-M1 and KI-ΔL2a homozygous mice were stimulated with anti-CD3ε antibody and 4 days later analyzed for CD8αα expression by staining with thymic leukemia (TL) tetramers. Percentages and MFIs of positive cells are shown within each profile box. Activated splenocytes from KI-ΔL2a mice displayed significantly increased ($p \leq 0.001$) CD8αα expression upon stimulation.

Briefly, the CD2 coding sequences serve as a surrogate reporter when appended directly to the basal CD8α promoter downstream of the E8i-C2 enhancer cassette in the presence or absence of L2a (Figure 7A).

Splenocytes were isolated from transgenic mice carrying either the L2a WT or the enhancer lacking L2a. Cells were stained with anti-CD11c (a pan DC antibody), anti-CD8α and anti-hCD2 for detection.

A representative analysis of gated cells is shown in Figure 7B. L2a-WT and L2a-D both expressed equivalent levels of the hCD2 reporter as the MFIs of CD8αα on CD11c+CD8αα+ DC and CD11c–CD8αα+ T cells were quite similar.

However, while its levels were significantly lower than CD11c–CD8αα+ T cells (~4-fold; $p \leq 0.01$), L2a-D clearly activated detectable levels of the hCD2 reporter in DC (Figure 7B). These data indicated that L2a directs expression of SATB1 in CD8αα+ splenic DC.

Its significantly lower levels suggest that SATB1 regulation of CD8αa expression in dendritic cell differs both quantitatively and qualitatively from that in CD8 T cells.

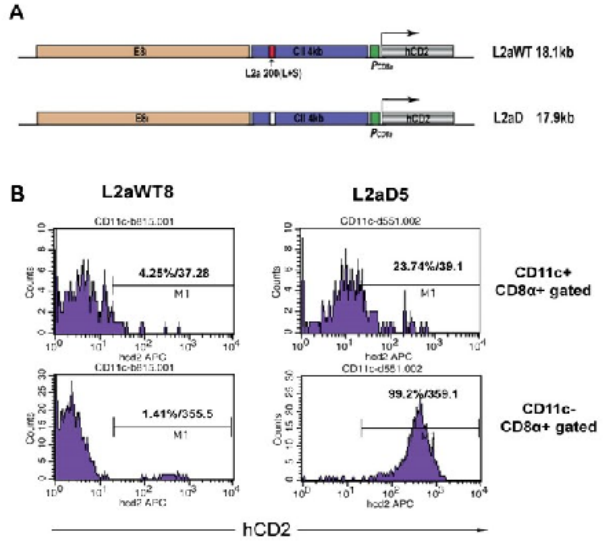


Figure 7. Splenic CD8αα$^+$ Dendritic Cell (DC) expression is modestly repressed by L2a in transgenic mice. A. L2a transgenic constructs. A human (h) CD2 reporter gene contains the mouse CD4 exon I, a portion of intron I lacking the CD4 silencer, and the untranslated portion of exon II (a CD4 splicing module) fused to hCD2 cDNA and an SV40 polyadenylation site. A PCR-amplified mouse CD8α promoter was inserted into the polylinker to make a construct termed Tg-α . The wild type construct (L2aWT) is a 4 kb DH cluster II fragment, and the L2aD construct carries a deletion of the 210 bp L2a sequence. Transgenic C57BL6 mice were created with each construct as previously detailed [27]. The number of integrated copies was determined by comparing signal intensities of WT and transgene-containing bands by Southern blot analysis of transgenic liver DNAs. We estimated that L2aWT employed in this study had 8 concatermized copies, and L2aD carried 5. B. Splenocytes isolated from L2aWT8 and L2aD5 mice were stained with anti-CD11c, anti-CD8α and anti-hCD2 antibodies. Gated cells were analyzed as shown. Percentages and MFIs of hCD2 positive subsets are shown.

KI- L2a but not KI-M1 mice are deficient in intestinal expression of CD8αα$^+$ DC

A recently identified enhancer, E8VI, that resides ~5 kbp telomeric to E8I (Figure 1A), was shown to direct expression of CD8αα DC-but not CD8αα IEL [17]. Those results and the data of Figure 7 prompted us to determine the effect of L2a loss on this subset in the intestine. Intestinal IEL were isolated and analyzed as outlined in Materials and Methods.

As shown in Figure 8A, the relative frequency of CD11c+CD8αα+ DC in KI-M1 mice, which bear mutations within the SATB1 binding site of E8i (Figure 2), was indistinguishable from that of KI-WT controls (20.4% *vs.* 27.5%).

However, when assayed in KI- L2a mice, which carry a 200 bp deletion of L2a that eliminate binding of both SATB1 and CDP/CUX1 (Figure 1B), the frequency of CD8αα expressing DC was substantially

decreased when compared to KI controls (8.8% KI- L2a *vs.* 27.46% KI; p ≤ 0.001; Figure 8B).

These observations suggest that either CUX1, or another as yet to be identified L2a binding factor within the L2a enhancer, is required for maximal CD8αα$^+$ dendritic cell function.

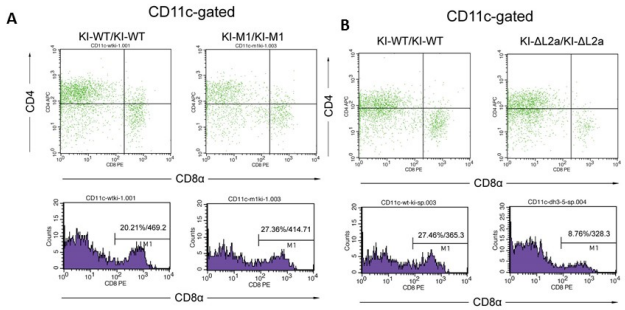


Figure 8. CD8αα expression is unaltered on KI-M1 yet significantly reduced on KI-ΔL2a CD8αα+ intestinal dendrite cells. The expression of CD8αα homodimers expressed on the intestinal subset of CD11c+ DCs was analyzed as shown in previous FACS figures. Percentages and MFIs of positive cells are shown. A. CD11c + DCs from KI-M1 mice showed no difference from KI-WT controls in expression of CD8αα (20.4% *vs.* 27.5%). B. Decreased CD8αα expression on dendrite cells of KI-ΔL2a mice. Percentages and MFIs of positive cells are indicated (/) inside the profile boxes. CD8αα expression on CD11c+ DCs from KI-ΔL2a mice is decreased ~3-fold (p ≤ 0.001).

Discussion

Previously we employed a transgenic approach to test whether L2a is the element within DH cluster II responsible for modifying the function of the E8I enhancer [27]. Our results identified L2a as a silencer of Cd8 transcription and further implicated SATB1 as a positive transactivator whose expression contributes to reversing the L2a-mediated silenced state. Here we report results of knock-in studies aimed at further investigation of the function of the L2a element in regulating Cd8α. This approach confirmed our earlier observations in conventional CD8 T cells, albeit repression was modest. However, we identified an unsuspected and more penetrant function of SATB1-L2a interaction in CD8αα$^+$ Intraepithelial Lymphocytes (IEL) and CD8αα$^+$ dendritic cells (DC).

L2a repression in conventional T cells

The M1 mutant knock-in mice, which carry mutated residues within the L2a binding site that eliminate SATB interaction (Figure 1A and 2D), showed no significant changes in T cell developmental markers nor in CD8 expression in thymocyte or in peripheral T cell subsets (Figure 3). Three out of five knock-in mice (KI-L2a), in which the entire L2a element was deleted, showed modest, yet statistically increased CD8SP expression in thymocytes but not in LNs (Figure 4). We observed ~2-fold elevation in CD8CD4 DP CD3 expression (Figure 4 B,C), suggesting that L2a-deficient thymocytes might be more activated and/or proliferative than WT controls. These results confirm, our findings in transgenic mice in which overexpression of L2a led to the same heterogeneous outcome [27].

We suggest that the observed heterogeneity owes to variegation-a phenomena resulting in silencing of a gene in some, but not all, cells *via* abnormal juxtaposition with heterochromatin or chromatin conformation [49]. Variegated expression of the Cd8 locus has been documented in several previous studies. For example, while targeted deletion of either E8I or E8II had no effect on CD8 expression in thymocytes or CD8 T cells [6,9], combined deletion of both enhancers resulted in variegated expression of CD8 in DP thymocytes and reduced CD8 expression in mature CD8 T cells

Crossing these mice to mice with conditional deficiency in DNA methyltransferase 1 [12] partially reversed variegation, suggesting a partial epigenetic block of CD8 expression due to deleted cis-acting elements. Loss of the TF MAZR, shown to negatively regulate chromatin modification of the E8II element [50], led to variegated CD8 expression in DP thymocytes [34]. Finally, double deletion of E8II and E8III led to mildly variegated repression of both Cd8α and Cd8β [35]. These observations suggest that loss of L2a results in a variegated phenotype that manifests either as a frank mutation, which appears to be the predominant outcome, or as a much less penetrant effect. Nonetheless, the results here are significantly less robust than those observed in transgenic studies [27]. This may owe to compensatory effects of other cis-acting elements present in the knock-in germline configuration but missing from the genomic transgenic locus.

SATB1 mediated repression of L2a IEL

IEL are found in epithelial layers of mammalian mucosa, including the gastrointestinal (GI) and reproductive tracts [37]. IELs provide protection at gut epithelial surfaces by regulating gut homeostasis, rapid responses to infection, and adaptive *vs.* innate immune responses without the need for priming [37]. IELs display either αβ or γδ TCRs (TCR) with the vast majority expressing the CD8αα coreceptor [36-39].

We observed a modest, yet consistent (p ≤ 0.6), trend of CD8αα homodimer enhancement on both TCRαβ+CD8β- and TCRγδ +CD8β- IEL following either full loss (KI-L2a) or SATB1 binding site mutation (KI-M1) (Figure 5). However, when these intestinal IELs were stimulated with anti-CD3ε antibody for 4 days and analyzed with thymic leukemia (TL) tetramers [39,41], we observed dramatic activation of CD8αα expression in both L2a-deleted KI- L2a (~8-fold; p ≤ 0.001; Figure 6A) and L2a-point mutated KI-M1 mice (~10 fold; p ≤ 0.001; Figure 6B). The data suggest that SATB1 acts cooperatively through L2a binding to repress expression of CD8αα in IELs.

Recently Sakaguchi et al. [17] observed equally robust stimulation of TCRαβ+CD8αα+ IELs that was dependent upon a newly characterized Cd8-associated enhancer termed E8VI (Figure 1A). E8VI, while shown to be bound by RUNX/CBFβ and BCL11b TFs, bears no EMSA-detectable or sequence homology-identified SATB1 binding sites (Figure 1B; data not shown). Thus, while SATB1 does not appear to contribute to E8VI-based transcription, it must be considered as a new addition to the TCR-CD8αβ/γδ and CD8αα regulatory network controlled by the CII enhancer (Figure 1A).

L2a repression of CD8αα$^+$ in splenic and intestinal dendritic cells

Murine DCs are classified into two lineages: Lymphoid and myeloid [51,52]. Based on expression of CD8αα homodimers, which originally were thought to be expressed exclusively on T cells, DC can be further divided into several additional subtypes [53]. These include CD8αα$^+$

DC, which lack the myeloid maker CD11b and were originally thought to develop exclusively from lymphoid-committed thymic T cell progenitors at low frequencies [54]. However, Traver et al. [48] demonstrated that both CD8αα+ and CD8αα- DC can be generated from common myeloid and lymphoid progenitors in mouse thymus and spleen. Their results suggested that CD8αα expression on DC reflects the differentiation or maturation status of DCs but does not indicate a lymphoid origin. While we observed no effect of SATB1 point mutation (KI-M) on CD8αα+CD11c+ DC isolated from gut (Figure 7A), robust reduction (~3.5-fold; $p \leq 0.001$) was achieved following complete SATB1 elimination (KI- L2a; Figure 7B). Again, we suspect that additional(s) factors deleted within the L2a 200 bp spanning region are responsible for this significant loss.

Conclusions and Extensions

There are multiple lineage-specific and stage-specific cis-acting elements involved in the regulation of Cd8 expression, suggesting a complex regulatory network of these closely linked elements. In contrast to its modest silencer function in unstimulated T cells and IELs, we found that L2a exerts a far more penetrant function in CD3-stimulated CD8αα$^+$ IELs and CD8αα$^+$ DC. Several of our findings implicating SATB1 in the context of the 125 bp mutation spanning the SATB1 binding site (KI-L2a), were not replicated in point-mutated KI-M1. We close here by considering one cis-acting and two trans-acting regulatory factors that might contribute.

The 12 bp palindrome (12-mer; S-Figure 2A) within the L2a INTER-LS region that separates SATB1 and CDP/CUX1 binding sites [27] is one such cis target. Introduction of a 2 bp substitution (termed NCO11; S-Figure 2A) into the palindrome (PAL) dramatically altered the binding pattern of SATB1 as well as CDP/CUX1 (S-Figure 2A, B).

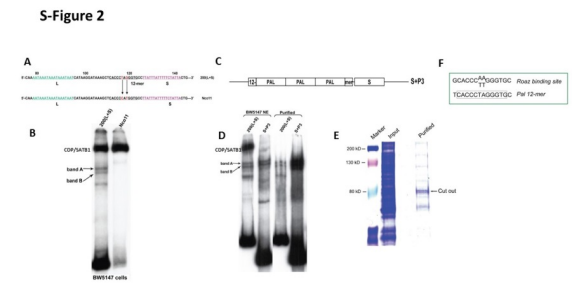
Two point mutations within the 12-mer abolished complexes (termed A and B) formed by unidentified proteins (S-Figure 2B). A trimerized 12-mer palindrome (S+P3; S-Figure 2C) was sufficient to capture these two proteins (S-Figure 2D) and was employed in affinity columns to purify them (S-Figure 2E).

Mass spectrometry (data not shown) identified one as PIGPEN, known as modulator of endothelial cell differentiation and angiogenesis [55]. The other (EST gi/26334035) is without ascribed function. But both have conserved Zn finger DNA binding domains.

A third candidate for a 12-mer binding protein, suggested by analysis of L2a using a transcription binding factor database (www.genomatix.de), was the OLF-1/EBF-associated zinc finger protein, ROAZ.

ROAZ binds to inverted repeats of GCACCC separated by 2 bp-sequences almost identical to the L2a 12-mer element (S-Figure 2F). A member of this family, EBFAZ/EV14 is essential for normal mouse B cell development but has yet to be characterized in other hematopoietic cells [56,57].

Finally, additional unpublished *in vitro* analyses (not shown) as well as previous studies [58,59] indicate that CDP/CUX1and SATB1 recognize the L and S motifs in fundamentally different ways and likely collaborate, since their binding sites, at least partially, overlap. To test this model directly, we are developing knock-in mice with targeted point mutations within the CDP/CUX1 L2a binding site.



S-Figure 2. Identification of two L2a palindromic 12-mer binding proteins. A. Upper: Schematic of L2a showing 12-mer (red), L region (red) and L region (magenta). Lower: Two point mutations (red) introduced into the 12-mer and constructed as an EMSA probe, termed Nco11. B. Schematic EMSA probe (termed L+P3) composed of a trimer of the 12-mer palindrome created by PCR. D. Purification of palindromic 12-mer binding proteins 1 and 2. EMSA probes noted at the top are the 200 bp spanning L2a and the L site palindromic trimer (L+P2). Left two lanes, unfractionated BW5147 extract; right two lanes, L+P3-Separose column affinity purified BW5147 extract. E. Coomassie stained SDS PAGE of proteins from (D) reveals two intensified bands. These were excised from the gel and determined by mass spectrometry to be PIGPEN [55] and EST gi/26334035 (no ascribed function). F. Comparison of the WT L2a palindromic 12-mer with the binding site of the ROAZ TF *via* sequence analysis using the **www.genomatix.de** database.

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Author Contributions

XY and HOT designed research; XY performed research; XY, GR and HOT analyzed data; XY, GR and HOT wrote the manuscript.

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