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STUDIES OF THE REGULATORY FUNCTION OF L2A IN MOUSE CD8 GENE EXPRESSION

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Dedication

I would like to dedicate this dissertation to Dr. Paul D. Gottlieb and his family.

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To my family for their support and constant encouragement.

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The TCR coreceptors CD4 and CD8 are crucial for thymocyte development and effector function of T cells. L2a was identified as a *cis*-acting DNA element putatively involved in CD8 expression. The L2a element has the properties of a nuclear matrix attachment region (MAR). It interacts with two MAR-binding proteins, SATB1 and CDP/Cux, through separated AT-rich regions, L and S. L2a mutants with an increased inter-LS region have decreased CDP/Cux binding, suggesting that both sites are required for binding at the same time. Upon binding of SATB1, these L2a mutants display altered DNase I hypersensitivity (DH) in the inter-LS region. A palindromic DNA 12-mer proximal to the S site was found to alter interactions between L2a and its binding proteins, and two 12-mer binding proteins have been identified.

Transgenic studies suggested that L2a is potential silencer for regulating CD8 expression. Transgenes driven by the L2a-containing DH cluster II and an enhancer $E8_I$ showed no reporter expression in thymic subsets or in peripheral splenocytes or in intraepitheal lymphocytes (IELs). Deletion of L2a resulted in

robust reporter expression, even in the DP population. A small fraction (1~5%) of the L2a-containing transgenic CD8SP thymocytes and peripheral T cells “escaped” L2a-silencing, suggesting that compensatory mechanisms can overcome silencing during transition to the CD8SP stage. Crossing this transgene onto a SATB1 knockdown background decreased the escape rate, indicating that SATB1 is involved in re-starting silenced CD8 expression.

Knock-in studies were carried out to further investigate the function of L2a. The M1 mutant knock-in mice, which have altered binding sites that abolish SATB1 interaction, showed no significant changes in CD8 expression. Knock-in mice in which the entire L2a element was deleted (ΔL2a) showed modestly increased CD8 levels in CD8SP thymocytes, peripheral CD8 T cells, and IELs. These effects are indicative of the consequences of losing a potential CD8 silencer, but their modest magnitudes suggest that other compensatory mechanisms suppress L2a function in the germline. Finally, targeted deletion of L2a resulted in significantly decreased CD8αα expression on splenic dendritic cells, implicating an unsuspected regulatory role for L2a in the lineage development of this myeloid sub-population.

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1. INTRODUCTION

1.1 T cell development and TCR coreceptors

1.1.1 An overview of T cell development

The development of thymocytes is a highly ordered and coordinated process. It provides a good model system for the study of regulatory mechanisms, and it has been used widely in the analysis of cell fate decisions and lineage commitment in vertebrates. Distinct developmental stages of thymocytes are defined by the expression of numerous regulatory components and cell surface molecules.

T cells mediate immune responses through cell surface T-cell receptors (TCRs). TCRs are composed of four invariant chains and two variable chains which form the interface to bind antigens [1]. Most T cells express TCR α and β variable chains. These cells develop in the thymus and recognize peptide/MHC class I or II molecules [2]. The majority of TCRαβ positive T cells also express CD4 and CD8 coreceptor molecules on their surface. The major functions of the coreceptors are enhancing adhesion and facilitating signaling through the TCR. They bind to the invariable regions of MHC class I or II molecules and interact with membrane-associated signaling molecules to facilitate TCR signal transduction [3-6]. CD4 is typically expressed on T helper (Th) cells, and it interacts with MHC class II molecules. CD8 is expressed on cytotoxic T cells and has specific interaction with MHC class I molecules. Therefore the CD4 and CD8 coreceptors are useful markers for T-cell sub-lineages and MHC restriction identification [7-11]. Both coreceptors are important for the recognition of antigen/MHC complexes by TCRs in both developing and mature T cells [12].

Developing thymocytes can be subdivided into four populations based on CD4 and CD8 coreceptor expression. At early stages, the thymocytes express neither CD4 nor CD8. These double-negative (DN, $CD4^-CD8^-$) thymocytes have functionally rearranged TCR-β chain genes. DN thymocytes transit to the double positive (DP, $CD4^+CD8^+$) stage during the rearrangement of the TCR-α chain gene and undergo an antigen-dependent selection process. Positive selection is accompanied by down-regulation of either CD4 or CD8, leading to single positive (SP, $CD4^+CD8^-$ or $CD4^-CD8^+$) T cells that express functional TCRs. Mature thymus-derived TCRαβ positive T cells express either CD4 or CD8 molecules which interact with antigen-presenting MHC molecules that are engaged with the TCR activation complex.

1.1.2 From DN to SP mature thymocytes

The immature DN cells constitute approximately 1-5% of total thymocytes, and they can be subdivided into several stages based on the expression level of the surface molecules CD117, CD44, and CD25. In brief, the thymic lymphoid progenitor cell ($CD117^+CD44^+CD25^-$) develops into the pro-T cell ($CD117^-CD44^+CD25^+$), then to the pre-T cell ($CD117^-CD44^-CD25^+$), and finally to the $CD117^-CD44^-CD25^-$ stage. A functional pre-TCR complex is formed at the pre-T cell stage due to the rearrangement and expression of the TCR-β chain gene. Signaling through this complex leads to the β-selection process and features proliferation and expansion of the thymocytes [13-16]. In addition to β-selection of DN thymocytes, pre-TCR complex signaling also leads to rearrangement of the TCRα gene and to the expression of CD4 and CD8 coreceptors. This results in progression of the DN cells to the DP stage.

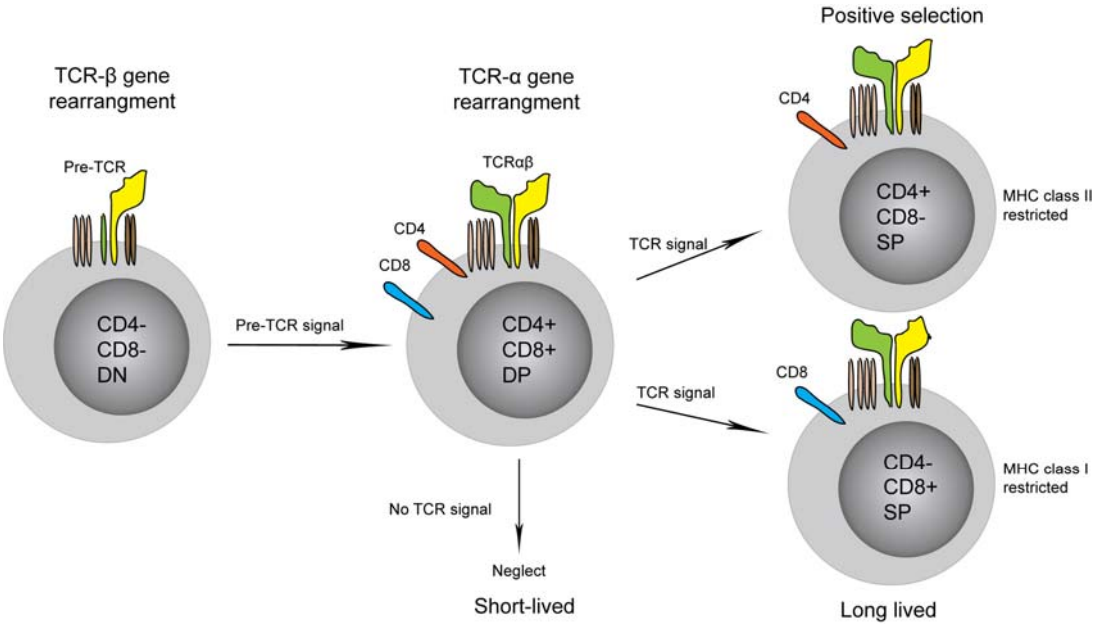

Fig.1. A simplified model of T cell development
Upon entering the thymus, thymocyte precursors rearrange their TCR-β genes as well as their TCR-γ and TCR-δ genes. Expression of the rearranged TCR-β allele forms part of the pre-TCR complex, which also contains CD3 components and the pre-T-α invariant chain, on the surface of DN thymocytes. After the stimulation of pre-TCR signals, DN cells differentiate into DP thymocytes, and they rearrange the TCR-α gene. A TCR-αβ complex, which contains TCR-α, TCR-β chains and CD3 components, is expressed on the surface of DP cells. Thymocytes with low avidity for self-peptide/MHC complexes die by neglect in the thymic cortex, and those with high avidity are eliminated by negative selection (not shown). DP cells with intermediate avidity for self-peptide/MHC complexes survive and undergo positive selection to differentiate in to mature CD8SP (MHC class I restricted) or CD4SP cells (MHC class II restricted). Figure adapted from Bosselut *et al*.[17].

About 80-90% of thymocytes are DP cells, and they express a mature TCRαβ receptor complex on the surface. The DP thymoctes undergo positive and negative selection [9] [18], and only a small portion of them develop into CD4SP or CD8SP mature thymocytes. During this process, the avidity of the TCR for intrathymic self-MHC/peptide complex determines which of the DP thymocytes will be rescued from apoptosis. Most DP thymocytes die within a few days of generation by neglect because their TCRs fail to be engaged and cannot mediate signal transduction [19] [20]. If the thymocytes express TCRs of high avidity for self-MHC/peptide complexes, they will be eliminated by TCR-induced apoptosis (negative selection). Therefore, only a few DP thymocytes that express a TCR complex capable of recognizing self-MHC/peptide complexes with appropriate avidity will be rescued from cell death and develop into CD4SP or CD8SP T cells [21-24] (Fig.1).

1.1.3 Mechanisms of lineage decision

The mechanism underlying CD4 and CD8 lineage commitment has been the subject of intense investigation over many years, and recent reports have begun to reveal the molecular details [25]. MHC specificity initially was linked to CD4/CD8 lineage determination by the instructive or the stochastic/selective, model [26, 27]. The instructive model proposed that the engagement of both the TCR and the coreceptor by MHC molecules directs CD4/CD8 lineage commitment [28, 29]. According to this theory, thymocytes are matched with their lineage and MHC specificity, which requires that signaling via MHC Class I specifically promotes positive selection and differentiation into CD8 T cells. Similarly, MHC class II signaling promotes CD4 T cell differentiation. In contrast, the selective model proposed that lineage choice is not dependent on TCR/MHC signals but is induced by other ligands. Thus, some of the

thymocytes are mismatched, but they are eliminated later because of lack of MHC co-engagement of TCR and the coreceptors [30-33].

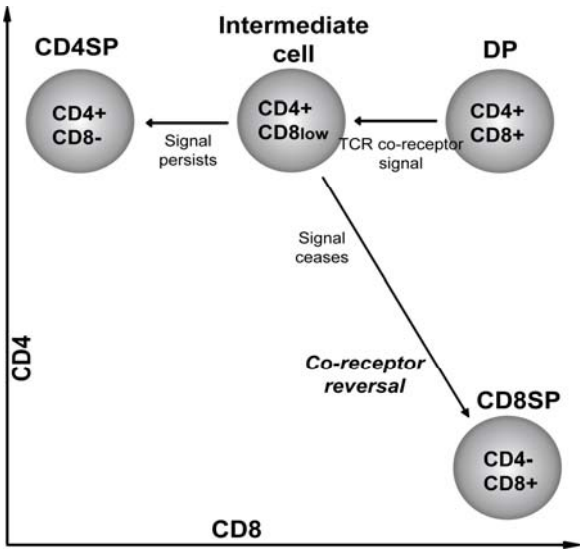

Fig.2. A schematic of the kinetic model of lineage decision
DP thymocytes are preprogrammed to respond to TCR coreceptor signals regardless of the MHC specificity. They convert into $CD4^{+}CD8^{low}$ intermediate cells by terminating *CD8* transcription. Continued signaling drives intermediate thymocytes to differentiate into CD4SP cells. Ceased signaling leads to "coreceptor reversal" and development into CD8SP thymocytes. Figure adapted from Singer [25].

Recent suggestions indicating that lineage commitment may be determined by signal duration, not signal intensity, have lead to two related signal-duration models [25, 34, 35]. One model proposes that the duration of the TCR signal instructs the fate of DP thymocytes; ie, prolonged signals drive DP thymocytes to CD4SP cells, and short-duration signals instruct them to differentiate into CD8SP cells. This model suggests that lineage commitment occurs in DP thymocytes before any changes in transcription or translation of the coreceptors. Alternatively, the kinetic signaling model postulates that lineage decision is influenced by differential regulation of coreceptor gene expression. All DP thymocytes transit into a $CD4^{+}8^{low}$ intermediate stage by transiently terminating CD8 transcription upon receiving intrathymic TCR co-receptor signals. These intermediate cells will develop into CD4SP cells if the signals persist. Cessation of TCR signaling will reinitiate CD8 gene transcription and terminate CD4 transcription. Thus, these intermediate thymocytes will differentiate into CD8SP cells, a process originally termed “coreceptor reversal” [34] (Fig.2).

Identification of the *cis*-acting regulatory elements and factors controlling CD4 and CD8 gene transcription has led to support of both models. Further studies on the transcriptional regulation of coreceptor genes are required to understand more about CD4/CD8 lineage differentiation.

1.2 Regulation of CD4 and CD8 coreceptor expression

1.2.1 *CD8* genes and proteins

The *CD8a* and *CD8b* genes reside 36 kb apart on mouse chromosome 6. The *CD8b* gene lies upstream of the *CD8a* gene and they are in the same transcriptional orientation [36]. These closely linked coreceptor genes have

partially overlapping but distinct expression patterns, which indicate they may be regulated both independently and coordinately.

The CD8 cell surface glycoprotein can be expressed as two isoforms: CD8αβ heterodimers or CD8αα homodimers [37, 38]. Thymus-derived T cells usually express CD8αβ heterodimer on their surface, whereas extrathymically derived intraepithelial lymphocytes (IELs) from the gut [39, 40] and $CD8^+$ dendritic cells (DCs) [41] express CD8αα homodimers.

CD8αβ is a disulfide-bonded heterodimer of two proteins, CD8α and CD8β, which are encoded by the *CD8a* and *CD8b* genes, respectively. By virtue of its interaction with a monomorphic determinant on the class I MHC molecule, the CD8αβ molecule functions as a corecepter for recognition of a target peptide/class I MHC complex by the αβ-TCR of a class I-specific T lymphocyte. In addition to increasing the avidity of the interaction, CD8αβ interaction provides a signaling function to T lymphocytes through the $p56^{lck}$ tyrosine kinase associated with the cytoplasmic tail of the CD8α subunit. Mice with a *CD8a* gene disrupted by homologous recombination lack class I MHC-specific cytotoxic T lymphocytes [42]. When the *CD8b* gene was disrupted [43] or its cytoplasmic tail was removed [44], mice expressed T cells with CD8αα homodimers, but they had abnormal negative and positive selection. Thus both CD8 subunits are important for the function of class I MHC-specific T cells and the development of thymocytes.

1.2.2. DNase I hypersensitivity and chromatin structure

In the eukaryotic nucleus DNA is associated with histones and is packaged into chromatin [45]. Chromatin is condensed into a 30 nm diameter fiber and is organized into nucleosomes, each of which consisting of 146 base pairs of DNA wrapped around a histone octamer core. The extent of chromatin

condensation is thought to be related to active and inactive states of gene transcription. A tightly condensed heterchromatin structure is most likely a silenced region of the genome, and this packaged structure is not accessible to transcription factors and enzymes. On the other hand, a decondensed euchromatin configuration is thought to be an active gene locus, and this chromatin structure is devoid of ordered nucleosomal arrays [46]. The transitional processes between closed and open chromatin configuration is termed chromatin remodeling. A number of studies have suggested that post-translational modifications play an important role in controlling the closed or open chromatin state. Such modifications include glycosylation, phosphorylation, methylation, acetylation, and ubiquitylation [47-49]. Histone-modification enzymes, such as histone acetyltransferases (HATs) and class I histone deacetylases (HDACs, play major roles in the chromatin remodeling process [50].

The chromatin structures associated with differential DNA accessibility are the basis of the DNase I hypersensitivity (DH) assay. This method is widely used to identify DH sites in an expressing gene locus in its natural chromatin configuration. The DH sites likely indicate the location of *cis*-acting elements, such as enhancers and promoters, in the vicinity of genes [51]. DNase I hypersensitivity assays have been used to identify some of the major CD8 *cis*-acting regulatory elements.

1.2.3 *cis*-acting elements in *CD8* gene regulation

Both DH site and transgenic reporter assays have been used to identify some of the major CD8 *cis*-acting regulatory elements. A long-range DH site assay revealed four (I-IV) DH site clusters in an 80 kb genomic fragment spanning the *CD8a* and *CD8b* genes in mouse [52] and six clusters covering 95

kb in human [53]. Using transgenes derived from P1 bacteriophage clones containing large genomic inserts (approximately 80 kb of DNA covering *CD8a* and *CD8b*), several individual *cis*-acting regulatory elements were further dissected functionally. Four enhancers (E8I to E8IV) were demonstrated to be required for *CD8* gene expression in CD8αβ T cells. They are located between the *CD8a* and *CD8b* loci and overlap with the DNase I hypersensitivity sites [54-60] (Fig.3). All four enhancers are CD8-lineage specific and are active at defined T cell developmental stages.

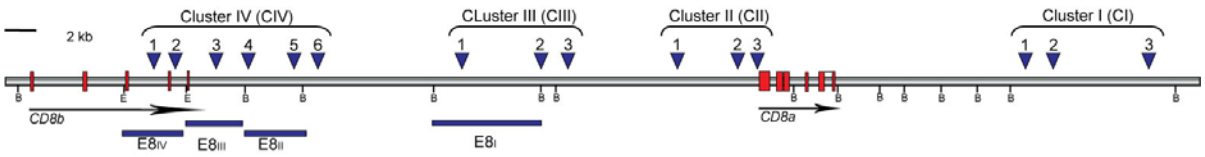

Fig.3. Map of the mouse *CD8a* and *CD8b* gene loci
A map of the mouse *CD8* gene locus showing four DNaseI-hypersensitivity (DH) clusters I to IV (CI-CIV). Triangles show DH individual sites. The horizontal bars denote the enhancers E8I, E8II, E8III, and E8IV. CIII-1,2 for E8I, CIV-4,5 for E8II, CIV-3 for E8III, and CIV-1,2 for E8IV. All BamHI (B) and relevant EcoRI sites are shown. Adapted from Kioussis *et al.* [60].

Ellmerier *et al*. [55] and Hostert *et al.* [54] described an enhancer covering DH sites 1 and 2 (HS-1 and HS-2) within DH cluster III (Fig.3), which resides approximately 16 kb upstream of the transcription initiation site of *CD8a*. This enhancer, termed E8I, regulates expression of the *CD8a* gene in mature CD8 T cells and in intraepithelial lymphocytes (IELs) of the intestinal wall. More

interestingly, the E8I enhancer is inactive in double positive (DP) thymocytes but becomes functional only after positive selection. In 1998, two groups, Ellmeier et al [57]and Hostert et al [56], studied, in more detail, the roles of sequences within these DH sites using a reporter transgene encoding the human CD2 surface antigen. In agreement with the earlier studies, they observed that the E8I enhancer directed expression of the hCD2 reporter in CD8SP thymocytes, CD8 peripheral T cells and CD8αα IELs of both TCRαβ and TCRγδ lineages, but not in double positive thymocytes.

Transgenic studies using genomic fragments derived from the cluster IV of DH sites revealed three other distinct enhancers (Fig.3). Enhancer E8II (CIV-4,5) (located in a 4.3 kb BamHI fragment) directed reporter expression in DP and CD8SP thymocytes as well as in mature CD8 T cells. The E8III enhancer (CIV-3) (a 4.1 kb BamHI fragment) is only active in DP thymocytes. The enhancer E8IV (CIV-1,2) (a 3 kb EcoRI fragment) directed expression of reporter gene in CD8 T cells and a subset of CD4 T cells [57]. All three of these enhancers are active only in thymus-derived T cells, but not in CD8αα IELs.

Some of the results described above suggested redundancy among these regulatory elements. Mice with targeted deletion of E8I (CIII-1,2) showed no effects on expression of *CD8a* and *CD8b* genes in thymus-derived T cells, but CD8 expression in IELs was eliminated [57]. This suggested that other *cis*-acting elements could compensate for the loss of E8I in thymus-derived T cells. Targeted deletion of either E8I or E8II had no effect on CD8 expression in thymocytes or CD8 T cells [56, 57]. However, the combined deletion of both enhancers resulted in variegated expression of CD8 in DP thymocytes and reduced CD8 expression in mature CD8 T cells [58]. An indistinguishable population of CD8-negative thymocytes was identified by surface markers and functional phenotypes from the whole DP thymocyte population.

Further studies demonstrated that deletion of both E8I and E8II enhancers lead to altered chromatin remodeling during T cell development [61]. Using chromatin immunoprecipitation and DNA methylation assays, the CD8-negative DP thymocyte population found in these mice was shown to have epigenetic modifications in the *CD8a-CD8b* locus, indicating an "off" state of chromatin. Crossing these mice to mice with conditional deficiency in DNA methyltransferase 1 (Dnmt1) [62] can partially revert the variegated CD8 expression, suggesting a partial epigenetic block of CD8 expression due to deleted *cis*-acting elements. In addition, a zinc finger transcription factor MAZR (Myc-associated zinc finger-related factor [63]) was found to interact with enhancer E8II and negatively regulate chromatin modification at *CD8* loci [61]. MAZR is highly expressed in DN thymocytes and is downregulated in DP and CD8SP thymocytes. MAZR can interact with the nuclear receptor corepressor N-CoR complex in DN thymocytes, and constitutive retroviral expression of MAZR led to variegated CD8 expression in DP thymocytes.

Enhancer E8III is an active *cis*-acting element only in DP thymocytes. Recently, a core 285-bp fragment was identified as sufficient for directing CD8 expression in DP thymocytes [64]. Further studies revealed that five elements within this fragment may contribute to full enhancer function [64]. Combined targeted deletion of the enhancer E8II and E8III in the mouse germline did not significantly change the expression levels of CD8α and CD8β in thymocytes or T cells [64]. Double-deficient mice had a small increase in CD4SP thymocytes, but $CD3^{high}CD5^{high}$ cells in this population decreased by about 10%. This suggested that double deletion of E8II and E8III leads to mildly variegated expression of *CD8* genes.

Another recent study indicated that the E8III enhancer may play a role in determining CD4/CD8 lineage choice during the positive selection process [65].

Transgenic mice expressing CD4 cDNA under the control of the CD8α promoter and E8III enhancer were bred to mice lacking endogenous CD4 expression. Thus, all CD4 expression in these mice was controlled by the E8III enhancer. The authors found that the E8III enhancer was inactivated by TCR-mediated positive selection signals. This might partially explain the early termination of CD8 gene expression in positive selected DP thymocytes (coreceptor reversal). Furthermore, the majority of MHC class II selected thymocytes developed into CD8 T cells with cytotoxic function in these transgenic mice. This might be a useful clue to understand the mechanism of CD4/CD8 lineage commitment.

DH cluster II was inactive in all transgenic reporter analyses, but deletion of CII-1,2 resulted in altered CD8 expression in both DP thymocytes and CD8 T cells [59]. These results are similar to those observed in E8I-E8II double deletion mice. Variegated CD8 expression in DP thymocytes was found in CII knockout mice, as characterized by a reduction in the CD8 expression level on a fraction of DP thymocytes compared to the remaining DP cells.

In summary, 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. Studies on the *trans*-acting regulatory factors interacting with them will be helpful in understanding the detailed mechanisms of CD8-lineage commitment.

1.2.4 Proteins regulating CD8 expression

Some nuclear protein factors play important roles in initiating and maintaining chromatin structure. They are expressed in various tissues and regulate expression of various genes, and generally they form large DNA-protein complexes which can bend the DNA backbone and alter chromatin

structure. HMG (High-Mobility Group) box-containing proteins have been reported to be involved in T cell development [66, 67]. This protein family promotes interactions between proteins that bind to sites that are far apart on the linear DNA sequences. HMG-mediated interaction brings the DNA-bound proteins closer together and stabilizes their DNA-protein complex [68, 69]. Two HMG proteins, lymphoid enhancer-binding factor 1 (LEF1) and transcription factor (TCF1), have been shown to bind to sequences of many lymphoid-specific genes. Deletion of LEF1 and TCF1 resulted in a block in thymopoiesis at the immature CD8SP stage [66, 67].

Another HMG protein TOX (thymus HMG-box protein) was suggested to affect lineage commitment of T cell development. Over-expression of TOX leads to an increased CD8SP thymocyte subpopulation and a reduced CD4SP thymocyte subpopulation, possibly as a result of direct or indirect effects on the *CD4* and *CD8* gene loci [70]. More direct evidence came from studies on the proteins of the BAF (BRG- or hBRM-associated factor) chromatin complex. The BAF complex contains the HMG-box protein BAF57, and the BRG1 ATPase. This complex has chromatin-remodeling activities similar to those of the SWI/SNF complex. Mutation in the HMG DNA-binding domain of BAF57 combined with mutated BRG1 leads to reciprocal regulation of *CD4* and *CD8* genes; ie, *CD8a* and *CD8b* are activated and *CD4* silencing is compromised [71].

Epigenetic regulation of T cell development has been demonstrated by studies of the protein factors involved in chromatin remodeling. Some DNA-binding regulatory factors bind to specific sequence in genes and recruit other factors to form chromatin remodeling machineries in the same region. This results in long-range and long-term changes in the chromatin, whose heritable structure can determine the expression levels of a target gene [72]. The nuclear

protein Ikaros has been shown to be a transcription factor involved in epigenetic regulation. Ikaros can interact with both histone deacetylases and the SWI/SNF complex to act as a repressor or an activator [73]. It was found that Ikaros interacts with the regulatory elements located in the *CD8a* and *CD8b* loci in CD8αβ cells [74], and deletion of the DH cluster II (CII-1,2), one of the Ikaros binding regions, resulted in variegated expression of *CD8* gene during DN to DP transition [59]. Furthermore, deletion of Ikaros or related family members led to impaired CD8α and CD8β expression [74, 75], and even resulted in impaired generation of B cells from multipotent haematopoietic progenitors [76].

1.2.5 Regulation of CD4 gene expression

The mechanisms of CD4 lineage differentiation are distinct from those of CD8 [11, 60]. Several T cell-specific enhancers have been identified within or close to the *CD4* locus, but none of them are CD4-lineage specific [77]. Instead of enhancers, a CD4 silencer, which is contained within a 434 bp fragment located in the first intron of CD4 gene, can repress CD4 expression in CD8 T cells and DN thymocytes [78, 79]. Deletion of the CD4 silencer resulted in variegated CD4 expression, characterized by CD4 expression in a random fraction of CD8 T cells and CD4 silencing in the remaining CD8 T cells [80]. A conditional knockout of this silencer demonstrated that it is not required for maintaining the silencing of CD4 expression in mature CD8 positive cells [81]. However this fragment is crucial in the establishment of CD4 silencing during CD8 thymocyte development, which remains silencer-dependent until the end of positive selection.

Additional functional studies of the CD4 silencer have identified several silencer-binding proteins, including RUNX (Runt-related transcription factor)

transcriptional regulators [82], SAF (silencer associated factor) [83], and the HES1 transcriptional repressor [84]. RUNX proteins have been characterized as CD4 silencing factors in CD8 lineage development. The RUNX family (RUNX1-3) of proteins contains a Runt DNA-binding domain, and all of them are expressed in the thymus. Results from knockout and conditional gene disruption experiments suggested that RUNX1 is actively represses CD4 transcription in DN thymocytes, and RUNX3 is mainly involved in CD4 silencing in CD8 lineage development [82, 85]. SAF has been shown to bind to the central region of the CD4 silencer, but mutants of SAF binding sites have no effect on CD4 silencing [80, 83]. The HES1 transcription factor is a target of Notch proteins [86], and it binds to the CD4 silencer to promote silencer activity *in vitro* [84]. The function of HES1 remains unclear as further studies conflicted as to its role in CD4 silencing and CD8-lineage development [80, 87].

1.2.6. CD8αα homodimer

The CD8αβ heterodimer is commonly expressed on thymocytes and CD8 conventional T cells which are MHC class I-restricted. In contrast, the expression of the CD8αα homodimer is not correspondent to MHC class I restriction of TCR [88, 89] and has been identified on various cell types, including IELs and dendritic cells (DCs) [90]. Previous cell culture transfection studies suggested that CD8αα can function as a TCR coreceptor [91]. However, it was later reported that CD8αα does not support the positive selection of conventional MHC class I-restricted T cells as effectively as CD8αβ *in vivo* [43, 92-94].

In the lumen of the intestine, there is a large population of various T lymphocyte subsets. These intraepithelial lymphocytes reside as single cells scattered among the epithelial cells with a number ratio of 1:4-9, making them

one of the largest T cell population in the body [95]. The majority of IELs express CD8αα on their surface. CD8αα has not been reported to be T cell lineage-specific or TCR-specific. It is expressed on TCRγδ IELs, and it can be co-expressed with CD8αβ or CD4 coreceptors on TCRαβ IELs.

There are two major subsets of IELs residing in the intestine. The first subset is the conventional IELs that express TCRαβ (MHC class I or II-restricted) with a TCR coreceptor, CD4 or CD8αβ. Some of these IELs can induce CD8αα expression during their translocation to the intestine, but they still maintain expression of CD4 or CD8αβ, making them triple positive [96, 97]. The second IEL subset expresses TCRαβ or TCRγδ mostly with CD8αα, but without the conventional coreceptors, CD4 or CD8αβ. These CD8αα IELs use the FcεRIγ chain of the CD16 complex of natural killer (NK) cells as part of the TCR-CD3 complex, and they can express various NK receptors [98]. Furthermore, the TCR-mediated selection and activation of CD8αα TCRαβ IELs differ from those in conventional TCRαβ IELs [88].

Recently the function of CD8αα on IELs has been partially revealed. Mucosal T cells can be induced to express CD8αα upon translocation to the intestine, which indicates that the induction of CD8αα may be an adaptation for the function and survival of T cell in the intestine [96, 99]. CD8αα has a specific and strong interaction with the thymic leukemia (TL) antigen, a non-classical MHC class I molecule constitutively expressed by the epithelial cells of the small intestine [96, 100]. The interaction between CD8αα and TL ligand leads to the activation of IELs, which is characterized by reduced proliferation and cytotoxicity, but increased cytokine production [96]. These antigen-stimulated responses are significantly different from those of activated peripheral CD8 T cells, which are characterized by clonal expansion and cytolytic activity.

The expression of CD8αα is not unique for IELs. It was reported that CD8αα can be co-expressed with CD8αβ on activated conventional T cells and some T cell leukemias [37, 38], although it is not expressed on resting peripheral T cells. Using cytotoxic T lymphocyte (CTL) cell lines activated via their TCRs *in vitro*, it was found that the expression of CD8αα is up-regulated, whereas CD8αβ is down-regulated and internalized [101].

More recently, CD8αα was reported to be transiently induced on a subset of conventional mature TCRαβ T cells after TCR activation [102]. This subset of CD8αα^{+} primary effecter cells expresses high levels of IL-7 receptors, which are commonly expressed on memory T cells and their predecessors. They can survive for a long time *in vivo* and differentiate into mature memory T cells [102]. CD8αα can not be induced in CD8α enhancer E8I knockout mice [58] during a primary response *in vivo* to lymphocytic choriomeningitis virus (LCMV). The knockout mice failed to generate CD8αβ memory T cells as well as secondary antigenic responses [102].

CD8αα can also be induced in adoptively transferred CD4 T cells during their migration to the intestine of the recipient mice [97]. Whlie *in vitro* stimulated human CD4 T cells can express CD8αα [103, 104], the role of CD8αα in CD4 T cell memory has not been demonstrated. Regardless of their MHC restriction and TCR specificity, the expression of CD8αα is dependent upon TCR activation, suggesting that CD8αα functions as a TCR modulator to regulate T cell survival and differentiation.

1.3 L2a as a *cis*-acting element that regulates *CD8a* gene expression

1.3.1 Identification of the L2a element

Carbone *et al*. reported that fusion of CD8 class I-restricted CTLs with the BW5147 thymic lymphoma resulted in CD8^{-} hybridomas [105]. In these

hybridomas, the *CD8a* alleles of the CTL fusion partner were CpG methylated, a state characteristic of $CD8^-$ cells. Previous studies in our lab showed that in such hybridomas, CD8 expression is shut-off at the level of *CD8a* gene transcription [106], whereas *CD8b* gene expression is not affected [107, 108]. Stable transfection of BW5147 cells with *a CD8a* gene reporter carrying differing lengths of 5' flanking sequences identified a putative regulatory element ~4.5 kb upstream as the target of negative regulation [106]. This DNA region is located within DH cluster II as defined by Hostert *et al*. [52].

Further studies revealed that a 220 bp region of this regulatory element, named L2a, has the properties of a nuclear matrix attachment region (MAR). Two MAR-binding proteins, SATB1 and CDP/Cux, bind to L2a through two AT-rich regions (called L and S) separated by a DNase I-hypersensitive region (referred to as the INTER-LS region) [106, 109] (Fig.4). DNase I footprinting indicated that SATB1 binds primarily to the L region and that CDP/Cux interacts with both L and S regions. A 12 bp palindromic sequence is located at the end of the INTER-LS region proximal to the S region. Further footprinting indicated that the binding of SATB1 to the L region results in a significant conformational change in the INTER-LS region. Binding of CDP/Cux with both L and S regions appeared to cause a more modest conformational change [109].

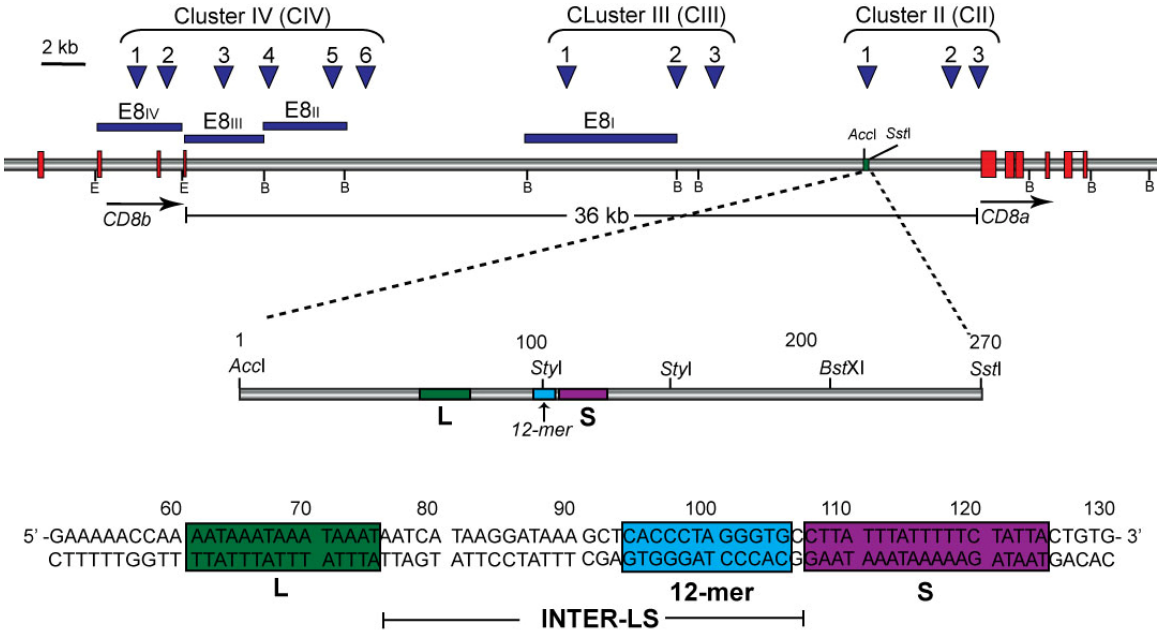

Fig.4. Schematic of the L2a element
The L2a element is located approximately 4.5 kb upstream of the mouse *CD8a* gene (upper map). The 270 bp AccI/SstI fragment is expanded to show the L, S, and INTER-LS regions identified in footprinting studies. The palindromic 12-mer is also shown. Adapted from Banan *et al.* [109].

1.3.2 Displacement switch model

Based on previous studies, a displacement switch model was proposed [109] to explain the interaction of SATB1 and CDP/Cux proteins with the L2a element (Fig.5). In the absence of SATB1, CDP/Cux binds primarily to the S region, and it also interacts with the L site via one or more of its multiple cut domains. The interaction of CDP/Cux to L and S sites may result in some distortion of the INTER-LS region closest to the S region. In the presence of SATB1, any CDP/Cux bound to the L region is displaced by binding of SATB1. The L region is the primary binding site of SATB1, and the binding of SATB1 to the L site creates a structural distortion in the INTER-LS region, particularly in the palindromic 12-mer sequence adjacent to the S site. Both the competition for the common binding site with L and the introduction of structural distortion has been demonstrated [109]. The binding of SATB1 and the DNA distortion might alter the affinity of L2a for the nuclear matrix, and thereby affect the transcriptional regulation of the adjacent *CD8α* gene. Interestingly, binding of SATB1 to the L2a element can specifically induce DNase I hypersensitivity in the palindromic 12-mer sequence. Because palindromes are frequently sites for protein interaction, it is possible that an unidentified protein can interact with the 12-mer and plays a role (together with CDP/Cux and SATB1) in the regulation of *CD8α* gene expression.

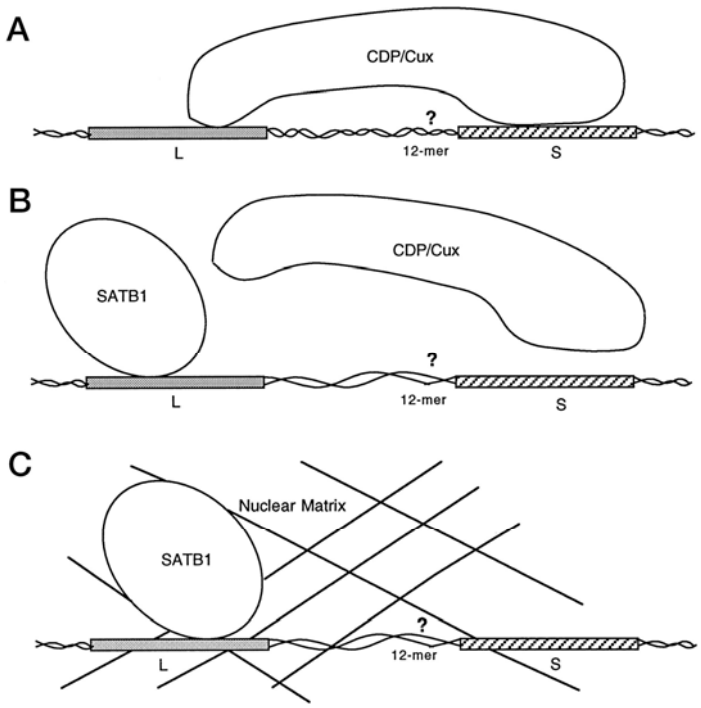

Fig. 5. Displacement switch model
A displacement switch model has been proposed to describe the interaction of SATB1 and CDP/Cux with the L2a element. CDP/Cux interacts with the S regions primarily and contacts the L region. Interaction of SATB1 with the L region leads to the displacement of CDP/Cux. SATB1 induces conformational changes in the INTER-LS region, which may affect the association of the L2a element with the nuclear matrix and favor *CD8a* gene transcription. Figure from Banan *et al* [109].

1.4 MAR regions and MAR-binding proteins

1.4.1 Nuclear matrix

In the eukaryotic cell nucleus, DNA is organized on at least two levels: into nucleosomes with histones and a 30 nm chromatin fiber, and by non-histone proteins which define and maintain looped domains 10 to 100 kb in length [110]. Extraction of nuclei with high salt solutions [111] or the chaotropic agent, lithium diiodosalicylate (LIS) [112], leaves behind a structure called the nuclear matrix or scaffold (also called nuclear cage, nuclear ghost, and nuclear core [113]). The nuclear matrix retains the same size and shape as the nucleus and consists of a proteinaceous network, whose contents differ depending on the preparation methods. Nuclear matrices typically consist of a nuclear lamina with residual nuclear pore complexes, residual nucleoli, and a filament network connecting the lamina with residual nucleoli [114].

Lamins have been known as the major components of the lamina in the nuclear matrix [115]. Further studies revealed that there are more than 200 different proteins included in the nuclear matrix, such as DNA topoisomerase II and nuclear mitotic apparatus protein (NuMA) [116, 117]. Additional nuclear matrix proteins have been identified by mass spectrometry [118]. The composition of the nuclear matrix differs among normal cell types and among normal and malignant cells. The latter difference has been useful in identifying cancer cell markers [119-121].

1.4.2 Nuclear matrix-associated DNA regions (MARs)

Nuclear matrices prepared by the LIS method contain matrix-associated DNA regions (MARs) which lie close to a number of different genes. MARs are thought to be the sites for the attachment of chromatin fibers to the nuclear matrix to form the 30 to 100 kb loop structures [122-125]. Generally MARs are

200-800 bp ATC-rich DNA regions and contain consensus sequences similar to topoisomerase II sites in *Drosophila* [126, 127]. MARs flank the kappa IgL and IgH enhancers [128, 129] and are located within transcription enhancers in three developmentally-regulated *Drosophila* genes [130]. MARs have been shown to be the *in vitro* binding sites for histone H1 and may affect histone H1-dependent chromatin repression [131, 132].

1.4.3 Regulatory function of MARs

MARs are defined by their capacity to mediate nuclear matrix association and can be grouped into two distinct classes [133]. One class of MARs may contribute to control of gene expression by forming structures or topological boundaries between distinct domains of eukaryotic chromosomes. Examples of chromosome domain boundary elements are the A elements flanking the chicken lysozyme gene [134], the specialized structures which flank the *Drosophila* heat shock locus and the insulator elements at the 5' end of the chicken β-globin locus [123, 135, 136].

The second class of MARs may function as intragenic control elements. One example are the MARs located within the immunoglobulin heavy and kappa light chain loci [128, 129]. These MARs are adjacent to intragenic enhancers or between the promoter and the enhancer. They generally do not contribute to transcriptional control in either transient or stable transfection assays in B lymphoid cell lines [137-140]. However, the MARs flanking the IgH enhancer function in transcription of the rearranged μ chain gene in B cells of transgenic mice. Furthermore, MARs were shown to enhance the acetylation of histones at the IgH enhancer distal nucleosomes [141]. The MARs appear to be required for broader reorganization of chromatin and to contribute to long range chromatin accessibility.

MARs have been shown to be important transcriptional regulators of chromatin remodeling [142], which is likely because most of the HDAC and HAT activities are associated with the nuclear matrix [143]. Moreover, MAR-binding proteins are involved in transcriptional regulation. For example, B cell regulator of IgH transcription (Bright) plays an important role in regulation of the IgH enhancer [144], and SATB1 can influence expression of a reporter gene flanked by MAR regions [145].

1.4.4 Special AT-rich Binding protein 1 (SATB1)

SATB1 was identified as a MAR-binding protein by Dickison *et al*. [146] from a thymic cDNA expression library screened with a concatamer containing the nucleation site for unwinding of the 3' MAR flanking the IgH enhancer. They reported that SATB1 is expressed abundantly in thymus and modestly in brain and testis. Other studies have identified SATB1 expression in other tissues [147] and in T lymphocyte cell lines [106, 109]. SATB1 specifically binds to the minor groove of A/T-rich regions of DNA in which one strand is also rich in C (ATC regions), and it is a component of the nuclear matrix [146]. Some of the AT-rich regions in MARs have a strong tendency to unwind by extensive base unpairing [148]. SATB1 binding is very specific, and it does not bind to AT-rich regions without unwinding ability, even if the region has a very similar sequence.

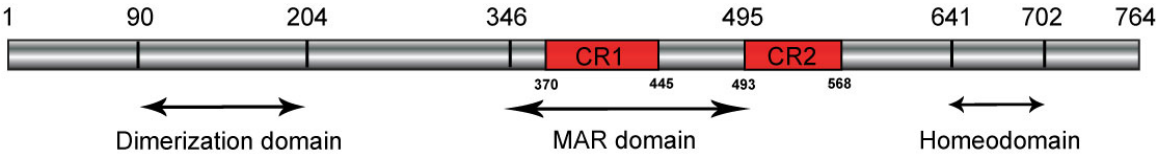

Fig. 6. Schematic diagram of mouse SATB1
Dimerization domain, MAR-binding domain, and homeodomain are indicated. Two Cut-like repeats, CR1 and CR2, are highlighted in red boxes. The numbers correspond to amino acid positions. Adapted from [149, 150].

To isolate genomic SATB1-binding sequence *in vivo*, cross-linked and *Sau3*AI-digested chromatin from Jurkat T cell lysates were immunoprecipitated with SATB1-specific antibody [151]. SATB1 bound DNA was cloned and sequenced, identifying 16 SATB1-binding sequences. Subsequent *in situ* hybridization experiments indicated that SATB1 binds to the bases of the chromatin loops. Further studies suggested that the chromatin anchoring to the nuclear matrix and loop formation by SATB1 are cell-type dependent [151, 152]. Thus, SATB1 may regulate tissue-specific gene expression by organizing higher order chromatin structure.

The murine SATB1 protein contains 765 amino acids and is 98% smilar to human SATB1 [153]. SATB1 contains a dimerization domain at the N-terminus, a MAR-binding domain in the middle, two cut-like repeats, and an atypical homeodomain at the C-terminus [149] (Fig.6). SATB1 was previously reported to bind DNA as a monomer, but subsequent studies suggested it functions as a homodimer. The dimerization domain is important for SATB1 to

bind to DNA. Truncated SATB1 protein without this domain has no DNA binding activity and is not functional, even thought it retained an intact homeodomain and MAR-binding domain [150]. The homeodomain does not bind DNA, but it cooperates with the MAR-binding domain associated with the core-unwinding element in the base-unpairing region [149].

A number of SATB1-interacting proteins have been identified. Using yeast two-hybrid screening of a Jurkat T cell cDNA library, SATB1 was found to interact via its dimerization domain with a novel variant of RNA polymerase II subunit 11 (RPB11) [154]. In pre-T lymphocytes, SATB1 was reported to colocalize with the X-linked lymphocyte regulated (Xrl) protein, which is also expressed in late stage B-lymphoid cell lines [155]. Another MAR-binding protein CDP/Cux was shown to bind SATB1, and both of them are repressors of the MMTV promoter. The interaction between SATB1 and CDP/Cux abolished DNA binding ability of both proteins, relieving the transcriptional repression at the promoter [156]. However, unpublished data from our lab was unable to confirm an interaction between SATB1 and CDP/Cux (Ingrid Rojas, personal communication).

Results from Kohwi-Shigematsu *et al.* [145] suggest that SATB1 may act as a negative regulator of gene expression. They stably transfected the BHK cell line, which expresses low levels of SATB1, with reporter genes containing or lacking flanking MARs. It appeared that SATB1 inhibited expression of the reporter containing the flanking MARs. SATB1 knockout mice are small in size, have disproportionately small thymi and spleens, and die at 3 weeks of age [157]. These mice exhibit neurological defects, such as an incomplete eye opening and clasping reflex. T-cell development was blocked mainly at the DP stage. The few peripheral CD4SP cells underwent apoptosis and failed to proliferate in response to activating stimuli [157]. Nearly 600 genes were

surveyed in SATB1-knockout thymocytes, and about 2% of them showed differences in expression [157]. RT-PCR results suggested that nine genes, including c-myc, had increased expression, which is consistent with the transcriptional repression shown by SATB1 previously.

Since deletion of SATB1 is lethal and has multiple effects on T cell development, Nie *et al* [158] constructed SATB1-reduced transgenic mice that are homozygous for a T cell-specific SATB1-antisense gene and heterozygous for a SATB1-null allele. These transgenic mice are significantly smaller than wild type mice, but they are generally healthy. In these mice, the thymic SATB1 protein level is significantly reduced, and there is a 3-fold reduction in CD8SP T cells in thymus, spleen, and lymph nodes. The composition of the thymus is similar to that of wild type with the exception of a slight increase in surface CD3 expression on CD8SP thymocytes. These results suggest an essential role for SATB1 late in the development and maturation of CD8SP T cells, possibly at the stage of coreceptor reversal [158].

Recent studies suggest that SATB1 interacts with proteins involved in chromatin remodeling. Using affinity purification, SATB1 was co-purified with the NURD (nucleosome remodeling and histone deacetylase) complex, which contains the ATP-dependent remodeling enzyme Mi-2, as well as HDAC1 and 2, mSin3A, and MTA-2 [159]. The NURD complex has been implicated in transcriptional repression of several genes. SATB1 can recruit ATP-dependent chromatin remodeling complexes that modify histone acetylation and nucleosome placement over long distances (~7kb) in the *IL-2Ra* gene [159]. Furthermore, SATB1 can form a three-dimensional network structure in mouse thymocyte nuclei. It was suggested that tethering of genes to the SATB1 network and orchestrated histone acetylation and methylation lead to gene activation or repression, depending on the locus [160].

1.4.5 CCAAT Displacement Protein (CDP)

The MARs flanking the IgH enhancer have been reported to have a negative regulatory function in T cells [161-163]. Sheuermann and Chen [162] identified a protein complex named NF-μNR, which binds to the MARs flanking the IgH enhancer. NF-μNR is present in T cells, macrophages, fibroblasts and early (but not mature) B cells. Wang et al [164] identified CDP/Cux as the principle component of this complex which mediates the negative regulatory activity attributed to NF-μNR. The CDP/Cux is the human (CDP, [165]) and mouse (Cux [166]) homologue of the Drosophila homeodomain protein, cut, which determines the cell fate of several embryonic tissue origins [167, 168]. Other members of this family include the rat CDP-2 protein [169] and the canine Cut-like homeobox factor (Clox) [170].

The CDP family constitutes a unique group of conserved homeoproteins among higher eukaryotes. The CDP/Cux proteins contain a single homeodomain and three cut repeats (Fig.7), and each of them is a highly conserved DNA binding domain. CDP/Cux also contains a coiled-coil leucine zipper (LZ) close to the N terminus and two active repression domains at the C terminus [171, 172].

Fig. 7. Schematic diagram of CDP/Cux
Coiled-coil leucine zipper (LZ) domain and homeodoman (HD) are indicated. Three cut repeats, CR1, CR2 and CR3, are highlighted in red boxes. Two active repression domains are shown at the C terminus. The numbers correspond to amino acid positions.

The homeodomain in CDP/Cux is a specific DNA-binding motif of 61 amino acids, which is encoded by a 183 bp DNA element, the homeobox. The homeodomain is important for substrate specificity. Proteins containing this domain function as transcriptional regulators of differentiation and development. The homeodomain folds into three alpha helices, and two of them form a helix-turn-helix (HTH) conformation, which is a common characteristic of transcriptional factors that can bind to the major groove of DNA [173, 174]. The third helix is the recognition helix that is responsible for DNA-binding specificity. A TAAT motif is conserved in almost all homeodomain binding sites, and homeodomain proteins prefer to bind these sites. The T at the 5' terminal is crucial for the interaction, because its mutation totally abolished homeodomain binding [175]. However, other binding sites with divergent DNA sequences have also been reported [175].

The cut repeats are approximately 70 amino acids in length and they have subtle differences in DNA binding specificity [165, 176, 177]. The cut repeats 2 and 3 bind to A+T rich DNA sequences, but they discriminate among similar sequences. Each of the three cut repeats have been shown to be independent DNA-binding motifs [176, 178] and prefer to bind to either CCAAT or ATCNAT sequences. As monomers, the individual cut repeats and the homeodomain do not interact with DNA very efficiently, but various combinations of cut repeats and homeodomains showed distinct DNA-binding specificity and kinetics [176, 178]. Compared with other transcriptional regulators, CDP/Cux appears to have greater flexibility in interacting with a wider spectrum of DNA sequences. In addition, the DNA-binding activity of CDP/Cux was reported to be regulated during cell cycle progression through its interaction with the core promoter of the cyclin kinase inhibitor, p21 [179].

CDP/Cux is evolutionarily conserved, and it is expressed from *Drosophila* to humans in various tissues. Originally CDP/Cux was identified as a transcriptional repressor, and a number of genes have been reported to be negatively regulated by CDP/Cux [180-186]. The CDP/Cux protein was reported to displace and compete for the binding of a CCAAT box-binding factor to negatively regulate the histone H2B gene in sea urchin [187]. CDP binding sites were found in the genes encoding human γ-globin [188, 189] and rat neural cell adhesion molecule (NCAM) [166]. CDP negatively regulates the human cytochrome gene, gp91-phox [190]. The repression mechanism of CDP/Cux may have a more general basis, because not all CDP/Cux binding sequence contain the CCAAT boxes [190].

Liu *et al.* [147] demonstrated that CDP/Cux and SATB1 binding to a negatively regulatory element within the long terminal repeat of the mouse mammary tumor virus (MMTV) inhibits viral replication. Chattopadhyay and

coworkers [191] reported that both SATB1 and CDP/Cux bind to a MAR located immediately upstream of the TCRβ chain enhancer. Similar to what we observed with the L2a element [109], the two factors bind to distinct yet partially overlapping sites [191]. They found that inclusion of this MAR region decreased reporter expression in transfected thymoma cell lines, and mice bearing a knock out of this MAR have no TCRβ chain transcription in developing T cells. Their results suggested that this TCR-associated MAR element may be the target of both positive and negative regulation and that CDP/Cux acts as the negative regulator.

CDP/Cux has been shown to repress gene expression by competing for binding site occupancy [184]. Generally, CDP/Cux competes with transcriptional activators for overlapping DNA binding sites to act as a repressor [165, 187, 190]. Furthermore, the C-terminal domain (CTD) of CDP/Cux is enriched in alanine and proline residues, which is a common property shared by many transcriptional repression domains. The cut and homeodomains of CDP/Cux were reported to interact with HDAC1 [182], and the direct recruitment of HDAC1 by CDP/Cux can mediate active repression of genes. The repression function of CDP/Cux has been reported to be regulated by post-translational modifications such as phosphorylation, acetylation, and proteolytic processing [179, 192-195].

In addition to acting as a repressor, CDP/Cux has been reported to activate transcription. The N-terminally truncated p110 CDP isoform was shown to stimulate DNA polymerase α promoter activity [196]. Cotransfection of CDP with the ITF2 factor induced tyrosine hydroxylase reporter gene activity [169]. Through interactions with different binding partners, CDP/Cux may have opposite functions on different promoters [197, 198].

To study the *in vivo* function of CDP/Cux, two different strains of CDP/Cux knockout mice have been generated. ΔC1 mutant mice express a truncated protein with a deletion of 246 amino acids including CR1, but have an intact C-terminus [199]. The ΔC1 protein retains DNA-binding activity and can translocate to the nucleus. The homozygous mice showed hair defects, and a high portion of pup loss was found in females. The other CDP/Cux knockout strain, ΔC, has a targeted deletion of the homeodomain [200]. The homozygous mice show neonatal lethality, but the heterozygous mice were healthy and fertile. CDP/Cux expression levels were significantly decreased in the thymus and mammary glands of heterozygous mice [201]. Some defects in T cells and B cells were observed, suggesting that CDP/Cux may play a role in lymphoid development.

1.5 Rational for this study

The *CD8a* gene encodes the CD8 coreceptor for the recognition of peptide/MHC class I complexes by developing T lymphocytes in the thymus and for mature peripheral CD8 T lymphocytes. CD8 T lymphocytes play an important role in fighting viral infection and destroying cancer cells.

Hostert and coworkers [56] constructed both the E8I enhancer and a 4.3 kb HindIII/HindIII fragment spanning DH cluster II into a transgenic reporter**.** Their results indicated that this combination not only directed reporter expression in CD8 cells, as expected of E8I, but also directed expression in DP thymocytes [56]. The DH cluster II alone was unable to direct reporter gene expression in previous studies [52]; yet it permits expression of a reporter gene in DP thymocytes in combination with the E8I enhancer**.** Furthermore, knockout of 3.4 kb spanning DH cluster II (CII-1,2), which contains L2a, caused variegated expression of the *CD8a* gene [59].

The observation that the cell type specificity of the E8I enhancer is extended to DP thymocytes by a DH cluster II fragment containing the L2a element is consistent with previous results [106, 109] that L2a and its binding proteins play a role in the regulation of *CD8a*. Based on these observations, we wanted to test whether the L2a element, which was reported to inhibit CD8α expression in transfection studies *in culturo* [106, 109], regulates E8I enhancer function and *CD8a* gene expression *in vivo*. Further, we wanted to determine if the L2a MAR in cluster II and its interacting proteins, SATB1 and CDP/Cux, are responsible for imparting DP thymocyte function to the E8I enhancer *in vivo*, or whether other sequences in cluster II were responsible.

It has been established that MARs are important for regulation of gene expression, but the mechanism underlying their function is not well understood. The effects of MARs and MAR-binding proteins on gene transcription may result from the association of a regulatory region of gene with the nuclear matrix — a situation that may favor transcription. By studying the involvement of the L2a MAR and its associated proteins, SATB1 and CDP/Cux, in *CD8a* gene regulation, we may be able to address the role of the nuclear matrix in the function of this element.

In addition to our observation that SATB1 and CDP/Cux interact with the L2a MAR region upstream of the mouse *CD8a* gene [109], three other groups have reported the binding of these two proteins to regulatory MARs [147, 191, 202]. In most instances, CDP/Cux appears to be a negative regulator, and some have suggested that SATB1 also has a negative effect on gene transcription [145, 147]. The binding of these two proteins to regulatory MARs is likely to be a general phenomenon, and the studies involving the L2a element may provide general significance in interpreting this.

Studying the function of the MARs and MAR-binding proteins may provide clues for understanding abnormal gene expression associated with cancer and inherited diseases. Since the L2a MAR and other MARs frequently contain binding sites for topoisomerase II, which is a target for many anti-cancer drugs, understanding how MARs and their associated proteins regulate gene expression will shed light on the basis for activity of anti-cancer drugs.

2. MATERIALS AND METHODS

2.1 Preparation of nuclear extracts

Nuclear extracts were prepared as described by Dignam *et al.* [203]. All steps were performed at 4^{o}C or on ice. 2x10^{8} cells were harvested and washed in PBS. The cells were resuspended in 5 ml buffer A (10mM HEPES [pH7.9], 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, and Protease inhibitor cocktail [Roche]) and incubated for 10 min. The cells were pelleted at 1,000g for 10 min and resuspended in 2ml of buffer A. A homogenizer with B pestle was used to lyses the cells (10 strokes), and the nuclei were pelleted at 1,000g for 10 min. The nuclei were washed with 2 ml of buffer A once and resuspended in 0.5-1.0 ml of buffer C (20mM HEPES [pH7.9], 25% glycerol, 0.42M NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM DTT, and Protease inhibitor cocktail), and homogenized with A pestle (20 strokes). Then the sample was magnetically stirred for 30 minutes, pelleted at 15,000g for 30 min. The supernatant was recovered and dialyzed against buffer D (20mM HEPES [pH 7.9], 20% glycerol, 0.1M KCl, 0.2mM EDTA, 0.5mM DTT, and Protease inhibitor cocktail) for 3 hours. After dialysis, nuclear extracts were centrifuged at 15,000g for 20 minutes, and supernatant was quick frozen in liquid nitrogen, and stored at -80^{o}C. Protein concentration was determined by Bradford assay.

2.2 Electrophoretic mobility shift assays (EMSAs)

Nuclear extracts (2-5µg) were mixed with poly-(dI-dC)(2µg) in binding buffer (20mM HEPES [pH 7.9], 20% glycerol, 0.1M KCl, 0.2mM EDTA, 10mM DTT, and Protease inhibitor cocktail). Binding reactions were done in 25µl total volume at room temperature for 5 minutes. After 20 min incubation with end-labeled probe (0.2ng), samples were electrophoresed at 120V for about 3 hours

through a 4% polyacrylamide gel (29:1) in 1xTBE buffer (90mM Tris-HCl [pH 8.0], 90mM boric acid, and 2mM EDTA). Gels were dried for 1 hour and autoradiographed for 4 hours using a phosphoimage screen or overnight using films with an intensifying screen at -80^{o}C.

For competition assays, unlabeled competitor DNA fragments were added to the reaction before the addition of radiolabeled probes. For antibody inhibition assays, 2µl of appropriate dilutions of antibody were added to the reaction before the addition of radiolabeled probes.

2.3 Preparation of probes for EMSA

All the probes were end-labeled with [α-^{32}P]dATPs using exo-Klenow enzyme. 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. Sequences of the primers are listed in Table 1 and Table 2.

Table 1. Primers used to make EMSA probes of E8III fragment.

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

Table 2. Primers used to make EMSA probes of DH cluster II.

Probe name	Primer Name	Sequence (5'-3')
L1	L2aEr4903F	CTC GAA TTC ATC TCC GCA AAC AGC AAG
	L2aBm5126R	TTA GGA TCC ACT GAC AGC AGA CAA C
L2	L2aEr5105F	GTC GAA TTC CAG GTT GTC TGC TGT CAG
	L2aBm5340R	TAT GGA TCC TGA CTG TCC TGG ACC TCA C
L3	L2aEr5505F	GTC GAA TTC ATT GCC AGC ATG ATG
	L2aBm5720R	ATA GGA TCC ATC TGT TGG TGG AAG C
L4	L2aEr5689F	GTC GAA TTC CTT CTC AAA CGC TTC CAC
	L2aBm5933R	TCT GGA TCC ATG GCT TGC CTG AAA CTC
L5	L2aEr5919F	GTC GAA TTC AGG CAA GCC ATG GCT AC
	L2aBm6156R	TAT GGA TCC TGA AGC AGG CAG CAG AG
L6	L2aHd6133F	AGT AAG CTT CTG CTC TGC TGC CTG CTT C
	L2aBm6368R	AGT GGA TCC ATG CAG TGA GCT ATA GC
L7	L2aHd6317F	AGT AAG CTT AGC TGC AAG ACT TGA AG
	L2aBm6582R	AGT GGA TCC TGG TCA CTG CTT CTC CTA C
L8	L2aHd6560F	AGT AAG CTT CCT GTA GGA GAA GCA GTG
	L2aBm6810R	AGT GGA TCC ATA TGG TGT GCA TGT GTG
L9	L2aHd6798F	AGT AAG CTT GCA CAC CAT ATG CAC AC
	L2aBm7024R	AGT GGA TCC TAT CTC ACT CAT GCC TC
L10	L2aHd7003F	AGT AAG CTT ATG TGA GGC ATG AGT G
	L2aBm7260R	AGT GGA TCC ATC AAG GTT GTG GTA TG
L11	L2aHd7240F	AGT AAG CTT CAT ACC ACA ACC TTG ATG
	L2aBm7489R	AGT GGA TCC TCG AAC TCA GAA ATC
L12	L2aHd7475F	AGT AAG CTT CTG AGT TCG AGG CCA G
	L2aBm7700R	AGT GGA TCC ATG GTA ATA GTT GAC TG
L13	L2aHd7665F	AGT AAG CTT ACA TCT AAG AGA TAC AG
	L2aBm7922R	AGT GGA TCC TGT CTC AGC ATA TAA AG
L14	L2aHd7906F	AGT AAG CTT TAT ATG CTG AGA CAG
	L2aBm8152R	AGT GGA TCC TAC TAT GGC TTC CAA AG
L15	L2aHd8135F	AGT AAG CTT GGA AGC CAT AGT AGG TAC
	L2aBm8407R	AGT GGA TCC TAC TCT TAA GAA TAC

2.4 DNase I footprinting

The DNase I footprinting of isolated bands was performed as described by Landolf *et al.* [204] and Banan *et al.* [109]. Before the footprinting, a series of titrations of DNase I was set up with DNA sample to optimize digestion conditions. Ten EMSA reactions were set up with appropriate probes and nuclear extracts. After 15 min incubation, DNase I (Ambion) was added to the reactions at optimized concentration, and samples were incubated at room temperature for 5 minutes. Samples were loaded on a 4% polyacrylamide gel (29:1) and electrophoresed at 120V for about 3 hours. The retarded bands and free probes were excised from the EMSA gel after wet exposure of two hours. The samples were eluted in 0.2M NaCl-TE by crush and soak method [146], phenol:chloroform extracted and ethanol precipitated with 100µg/ml yeast tRNA. The dried DNA samples were resuspended in loading dye (1:2 0.1M NaOH:formamide (v/v), 0.1% xylene cyanol, 0.1% bromophenol blue) according to the radioactive counts. Samples with equal counts were electrophoresed through 8% sequencing gel. The markers were produced by PCR using DNA sequencing kit (Promega).

2.5 Nuclear matrix binding assays

Preparation of nuclear matrix was carried out as described by Reyes *et al.* [205]. Nuclear matrix was washed three times in 1 ml of wash buffer (10mM Tris-HCl [pH7.4], 50mM NaCl, 1mM KCl, 0.25M sucrose, 0.25mg/ml BSA, and Protease inhibitor cocktail), pelleted at 10,000 rpm, 1 minute at 4^{o}C, and resuspended in 100µl of assay buffer (10mM Tris-HCl [pH7.4], 50mM NaCl, 2mM EDTA, 0.25M sucrose, 0.25mg/ml BSA, 150µg/ml salmon sperm DNA, and Protease inhibitor cocktail). After incubation for 30 minutes at room temperature on an orbital shaker, 20,000 cpm of probe was added and reaction

was incubated for 90 minutes at room temperature with shaking. The sample was washed two times with final wash buffer (assay buffer less the protease inhibitor) and pelleted at 10,000 rpm for 10 minutes at 4^{o}C. The pellet was resuspended in 50µl of TE buffer containing 0.5% SDS and 0.4mg/ml of proteinase K and incubated at 37^{o}C overnight. 100µg/ml yeast tRNA was added to the sample and the mixture was phenol:chloroform extracted and ethanol precipitated. The DNA pellet was dissolved in DNA loading dye and separated on 4% polyacrylamide gels.

2.6 Affinity chromatography

Self-ligated PCR-amplified S+P3 probe was coupled to CNBr-activated Sepharose 4B beads (Amersham Pharmacia) to make the DNA affinity column. Nuclear extract prepared from BW5147 cells was diluted and passed through the uncoupled column first to trap non-specific protein binding, and then twice through the S+P3 column. The proteins that bind to S+P3 column were eluted with buffer of higher salt concentration.

2.7 Cell lines

The T cell lines VL3.B4 (from Dr. I. Weissman, Stanford University) and BW5147 were grown in DMEM media supplemented with 10% fetal bovine serum (FBS). 1200M T cell line (from Dr. Ellen Richie, M.D. Anderson Cancer Research Center) was cultured in RPMI media supplemented with 10% FBS. 293T cells were cultured in DMEM media with 10%FBS.

2.8 Transient transfection of cultured cells

293T cell transfections were carried out using Fugene 6 (Roche) following the product instruction. T cell lines were transfected by electroporation.

Briefly, $5x10^6$ cells were washed and resuspended in 500µl PBS or culture media without FBS. Cells were mixed with 10µg of plasmid DNA and incubated at 4^oC for 10 minutes. Electroporation was carried out with a Gene Pulser (Bio-Rad), which was set at 950µF and 300V (for BW5147 or VL3 cells) or 220V (for 1200M) cells. Cells were incubated on ice for 10 minutes after the electroporation, and 10-15 ml of media were added.

2.9 Dual luciferase assays

L2a luciferase (Firefly) constructs were co-transfected with *Renilla* luciferase vectors into desired cell lines. 36-48 hours after transfection, cells were washed with PBS and resuspended in Passive Lysis Buffer (Dual-Luciferase Reporter Assay System, Promega). The cell lysates were applied to dual-luciferase assay following product instruction. The Firefly intensities were normalized by *Renilla* intensities to obtain the relative activities.

2.10 Production of transgenic mice

The L2a transgene construct was cut out of the vector using NotI, and an 18 kb DNA fragment was purified for pronuclear microinjection. The C57BL/6 zygotes were chosen to make transgenic mice to obtain an inbred background and save time from backcrossing. After the microinjection of transgene construct, the zygotes that survived injection were cultured overnight and those that developed to 2-cell embryos were transferred into the oviducts of 0.5-dpc pseudopregnant female mice. Southern blot was used identify positive founders that developed from injected zygotes. Founders were bred to C57BL/6 to obtain transgenic progeny that were used to analyze transgene expression.

2.11 Generation of L2a Knock-in mice

The knock-in constructs (30μg) were linearized by NotI 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 (200μg/ml) after one day. Ganciclovir was added (2μM) 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.

2.12 Preparation of genomic DNA from mouse tails

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

2.13 Southern blot analysis

The purified genomic DNA (25µg) 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 2xSSC, 0.1% SDS at 55^{o}C for 5 min, then washed twice with 0.1xSSC, 0.1% SDS solution at 55^{o}C for 15 minutes. Blots were air dried briefly and exposed using a phosphoimaging cassette. The [^{32}P] labeled probes were generated by random primer synthesis using a decaprime DNA labeling kit (Ambion)

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2.14 Isolation of cells from mouse thymus, lymph nodes and spleen

Thymus, lymph nodes and spleen were removed from euthanized mice and placed into 60mm 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 (0.5M NH_4Cl, 0.15M Tris-HCl [pH7.65]) for 5 minutes at room temperature. Cells were washed with HBSS and ready for desired treatment and analysis.

2.15 Isolation of IELs

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 30ml of RPMI for 45 minutes at 37^{o}C with low speed rotation to release the IELs. The RPMI medium containing released IELs was passed through a 70 micron cell strainer to filter out debris. Cells were centrifuged

(1,000rpm, room temperature) and resuspended in appropriate volume of RPMI medium. Cells were then purified with Ficoll-Pague Plus (Amersham) centrifugation (2,000rpm, 30min, room temperature), and washed with HBSS buffer.

2.16 FACS staining

Isolated cells were washed with HBSS (Sigma) buffer twice at 1,000rpm 4^{o}C, and resuspended in Hanks buffer (HBSS with 2% FBS and 0.1% sodium azide) on ice. Cells were counted and 1x10^{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 minutes, cells were stained with the desired antibodies for 45 minutes. Following two washes with 1ml Hanks buffer, 1ml 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 minutes after the wash steps of first staining. Cells were then washed and analyzed on a BD FACScalibur using CellQuest Pro software.

2.17 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.

2.18 Pronase treatment

Pronase treatment was carried out as described by Suzuki *et al.* [206]. Magnetically sorted CD8 positive splenocytes were washed in PBS and resuspended in PBS containing 0.01% pronase (Sigma) at a concentration of $2x10^6$/ml. Cells were incubated for 15 minutes at 37°C, washed with PBS, and treated with pronase solution for another 15 minutes at 37°C. Pronase activity was quenched by an equal volume of FBS, and cells were washed and transferred to 12-well plates. Treated cells were incubated for 18 hours at 37°C or 4°C. Viable cells were harvested and analyzed by FACS.

2.19 *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 $2.5x10^6$/ml and 2ml/well. Anti-CD3ε antibody (BD Biosciences) was added to the cells to a final concentration of 1μg/ml. Cells were sampled at day2 or day4 and applied to FACS analysis.

3. RESULTS

3.1 Studies on *in vitro* interactions between L2a element and its binding proteins

The L2a element has been identified as a MAR and interacts with two MAR-binding proteins, SATB1 and CDP/Cux [109]. Since they are both transcriptional regulators, the association of SATB1 and CDP/Cux with L2a is thought to be involved in its regulatory function of *CD8* gene expression. Both proteins have distinct but partially overlapped binding sites in the L2a sequence, and a "switch displacement" model has been proposed to explain the interaction among L2a, SATB1, and CDP/Cux [109]. Various mutants of the L2a element were constructed to study the mechanism in more detail.

3.1.1 Distance between L and S regions affects the binding of CDP/Cux to the L2a element

Previous results of DNase I and missing nucleotide footprinting experiments suggested that CDP/Cux binds primarily to the S region of L2a element, although it can also interact with the L region [109]. Furthermore, a mutant containing a deletion of the S region totally abolished the binding of CDP/Cux to L2a [109]. It was proposed that CDP/Cux binding requires both the L and S regions. This unique binding pattern may be the key in understanding the basis for the displacement of CDP/Cux by SATB1 at the L2a element. To further test this, mutants with an elongated INTER-LS region were constructed and evaluated in DNA-protein interaction assays.

To increase the length of INTER-LS region, one or more small DNA inserts were introduced between the L and S regions. An AflII restriction site was introduced immediately after the L region using GeneEditor *in vitro* Site

Directed Mutagenesis System (Promega). This mutation was made proximal to the L region to avoid interfering with the 12-mer palindromic sequence next to the S site. Two complimentary GC-rich oligos flanked by two AflII ends were annealed to make a 22-bp GC insert. The GC insert, introduced into the AflII site, was termed GC-1 (for one GC insert). Mutants GC-2 and GC-4 were made by inserting 2 and 4 GC inserts at the same site, respectively. Fig. 8A shows the mutated L2a constructs with GC inserts.

EMSA assays were performed with BW5147 nuclear extract and either radiolabeled wild type L2a 200(L+S) probe or mutant (GC-1, GC-2, or GC-4) probes (Fig. 8B). BW5147 is a CD4 CD8 double-negative thymoma cell line that expresses CDP/Cux but not SATB1 protein (data not shown). EMSA results showed that, with increased number of GC inserts, the binding of CDP/Cux to the probes became weaker. This suggested that both L and S regions may be needed for CDP/Cux binding. When the distance between the L and S regions became longer, perhaps CDP/Cux could span both sites at the same time. Association with only one binding site may result in weak binding. Furthermore, even the longest insert (GC-4) could not totally abolish binding, suggesting that intact, individual S and L sites interact with CDP/Cux but cannot collaborate to provide strong binding.

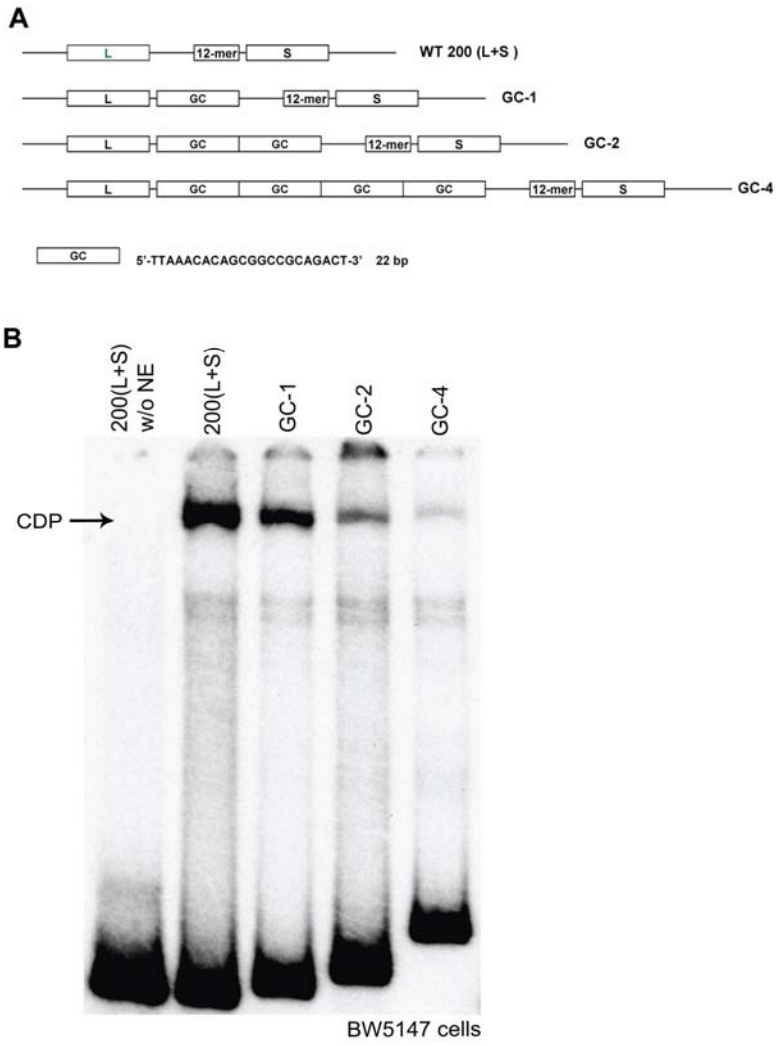


Fig. 8. L2a mutants with GC inserts show altered binding of CDP/Cux.
A. Wild type L2a element 200 (L+S) and mutants with GC inserts. GC-1, one GC insert; GC-2, two GC inserts; GC-4, four GC inserts. **B.** EMSA performed with BW5147 nuclear extract, radiolabeled 200(L+S) probe and mutated probes. The arrow indicates CDP/Cux complexes.

The L2a element has been shown to be a nuclear matrix associated region (MAR) [109]**.** MARs are mostly AT rich DNA sequences. Therefore, to eliminate the possibility that the GC content of the inserts employed above may affect the binding activity of CDP/Cux, L2a mutants with random sequence inserts were created using a similar method. Mutants RD-1, RD-2, and RD-3 have one, two, and three 22-bp random inserts respectively (Fig. 9A). EMSAs were performed with BW5147 nuclear extract and radiolabeled 200(L+S) probe, or mutant probes with random inserts (Fig. 9B). A decrease in binding was observed when the number of RD inserts increased. This result is consistent with that obtained from EMSA performed with GC inserts. Both GC inserts and random inserts seem to have the same effects on the binding of CDP/Cux to the L2a element. Thus, the change in length, but not content, of the DNA sequence is the main reason underlying these observations.

To confirm the previous EMSA results, competition EMSAs were performed using unlabeled GC mutant probes. As shown in Fig. 10, unlabeled probes differed in their abilities to compete with the wild type probe for CDP/Cux binding. These results indicate that probes with longer GC inserts have weaker competition than the wild type probe; ie, the bigger the insert, the weaker it competes relatively to wild type probe. Competition EMSA was also performed with unlabeled mutant competitor probes with RD inserts, and the results (Fig. 11) were consistent with that obtained with the GC mutants. These competition assays support the previous EMSA results, suggesting that the distance between L and S regions can affect the binding activity of CDP/Cux to the L2a element.

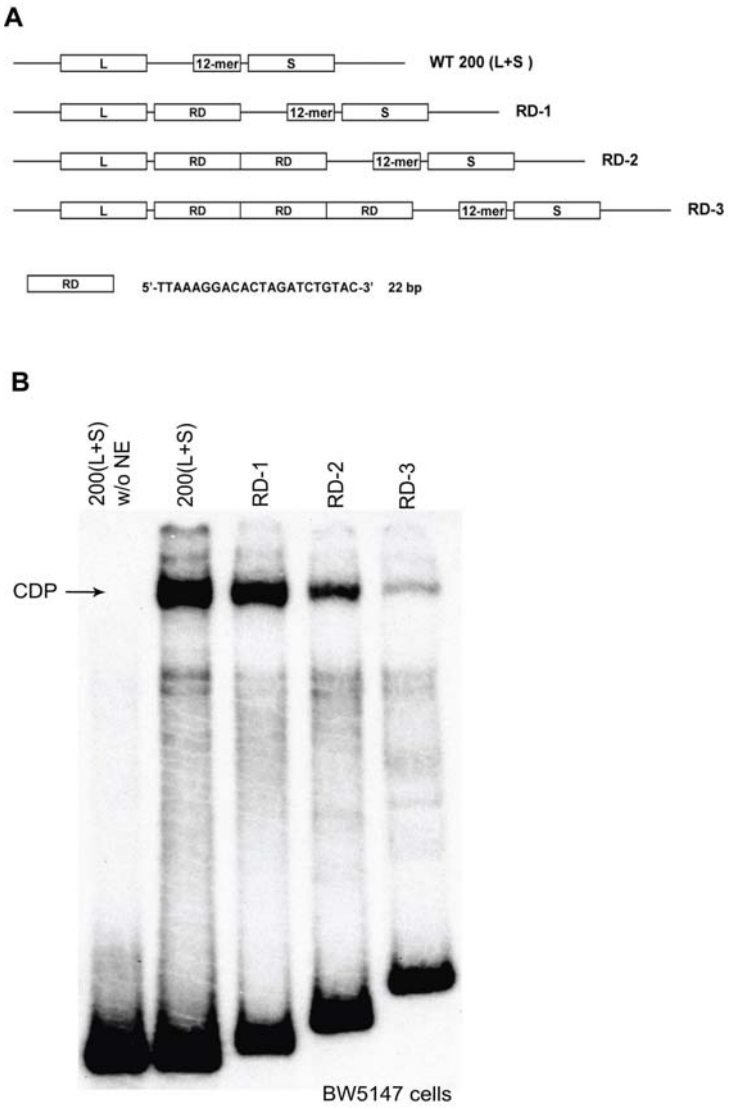


Fig. 9. L2a mutants with random (RD) sequence composition inserts show altered binding of CDP/Cux.
A. Wild type L2a element 200 (L+S) and mutants with RD inserts. RD-1, one RD insert; RD-2, two RD inserts; RD-3, three RD inserts. **B.** EMSA performed with BW5147 nuclear extract, radiolabeled 200(L+S) probe and mutated probes. The arrow indicates CDP/Cux complexes.

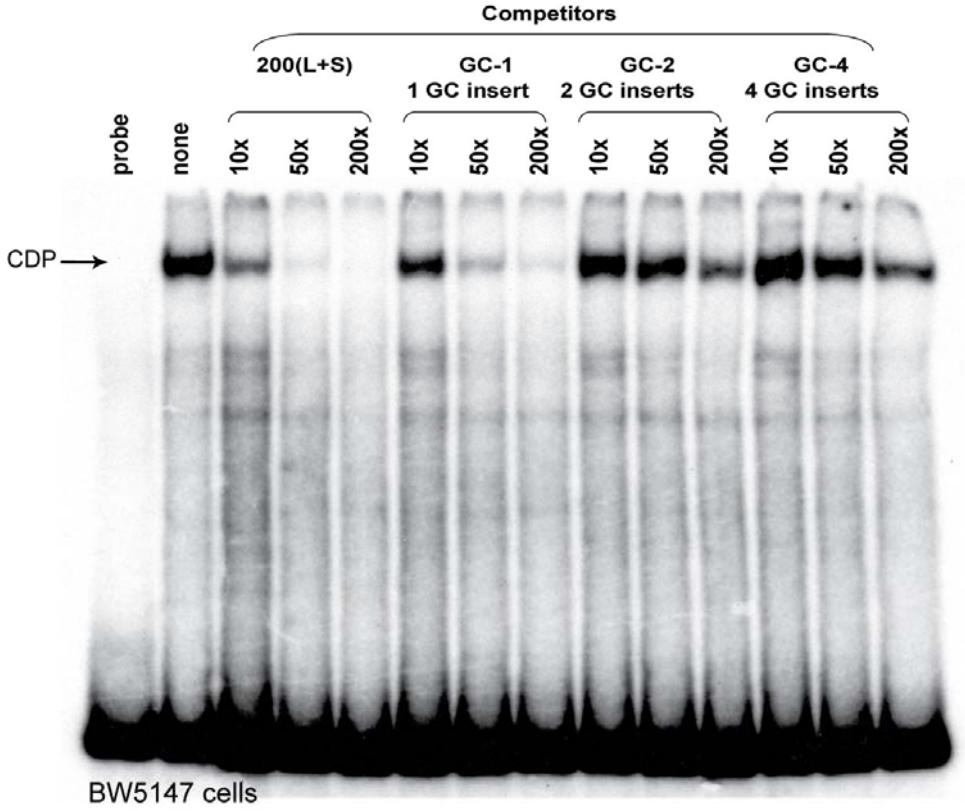


Fig. 10. Competition EMSA indicates weaker binding of CDP/Cux to mutant probes with GC inserts.
Competition EMSA performed with BW5147 nuclear extract, radiolabeled 200(L+S) probe, and unlabeled mutated GC-1, GC-2, GC-4 competitors. 10-200 molar excess of competitors were added after the addition of radiolabeled probe. The arrow indicates CDP/Cux complexes.

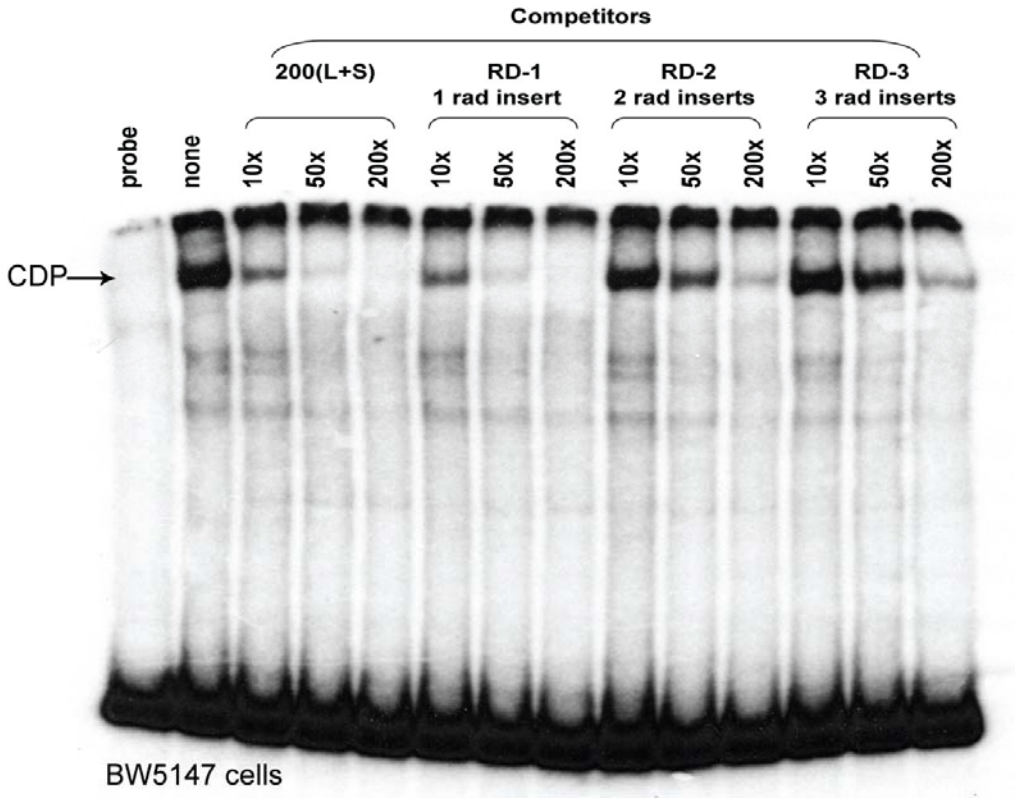


Fig. 11. Competition EMSA indicates weaker binding of CDP/Cux to mutant probes with RD inserts.
Competition EMSA performed with BW5147 nuclear extract, radiolabeled 200(L+S) probe, and unlabeled mutated RD-1, RD-2, RD-3 competitors. 10-200 molar excess of competitors were added after the addition of radiolabeled probe. The arrow indicates CDP/Cux complexes.

DNase I footprinting assays were employed to study how the binding of CDP/Cux to the L2a element decreased after the distance between L and S regions was changed. Fig. 12 shows the footprinting results performed with BW5147 nuclear extract and either 200(L+S) or mutant (GC-1 or GC-2) probes. Protection was observed of both L and S regions, and when the numbers of inserts in the INTER-LS region were increased, protection of both L and S regions decreased. This result is consistent with the EMSA results performed with BW5147 nuclear extract. The GC-4 probe was not used for footprinting because its EMSA complex with CDP/Cux was too weak to be isolated from the gel.

3.1.2 SATB1 shows a different binding pattern to mutant probes

To further characterize the interactions among CDP/Cux, SATB1 and the L2a element, nuclear extract from the VL3 cell line was utilized in EMSAs. VL3 is a CD8 positive T cell tumor cell line, which expresses both CDP/Cux and SATB1 proteins. Fig.13 shows an EMSA performed with BW5147 or VL3 nuclear extract, and radiolabeled 200(L+S), 300(L+S) probe or mutated GC probes. The wild type 300(L+S) was obtained from a different restriction digestion (PvuII) and was used as a control since it is the same size as GC-4. When using the VL3 nuclear extract, the SATB1 complex was super-shifted as the size of the probe increased. It appeared that the separate complexes for SATB1 and CDP/Cux merged into one complex when GC-4 was used as a probe. The 300(L+S) and GC-4 probes produced distinctly different CDP/Cux and SATB1 binding patterns. Thus, the size of the probes is not the reason why the binding pattern is changed. Instead, the results from VL3 nuclear extract suggest that the inserts in the INTER-LS region affect the binding activities of both SATB1 and CDP/Cux proteins.

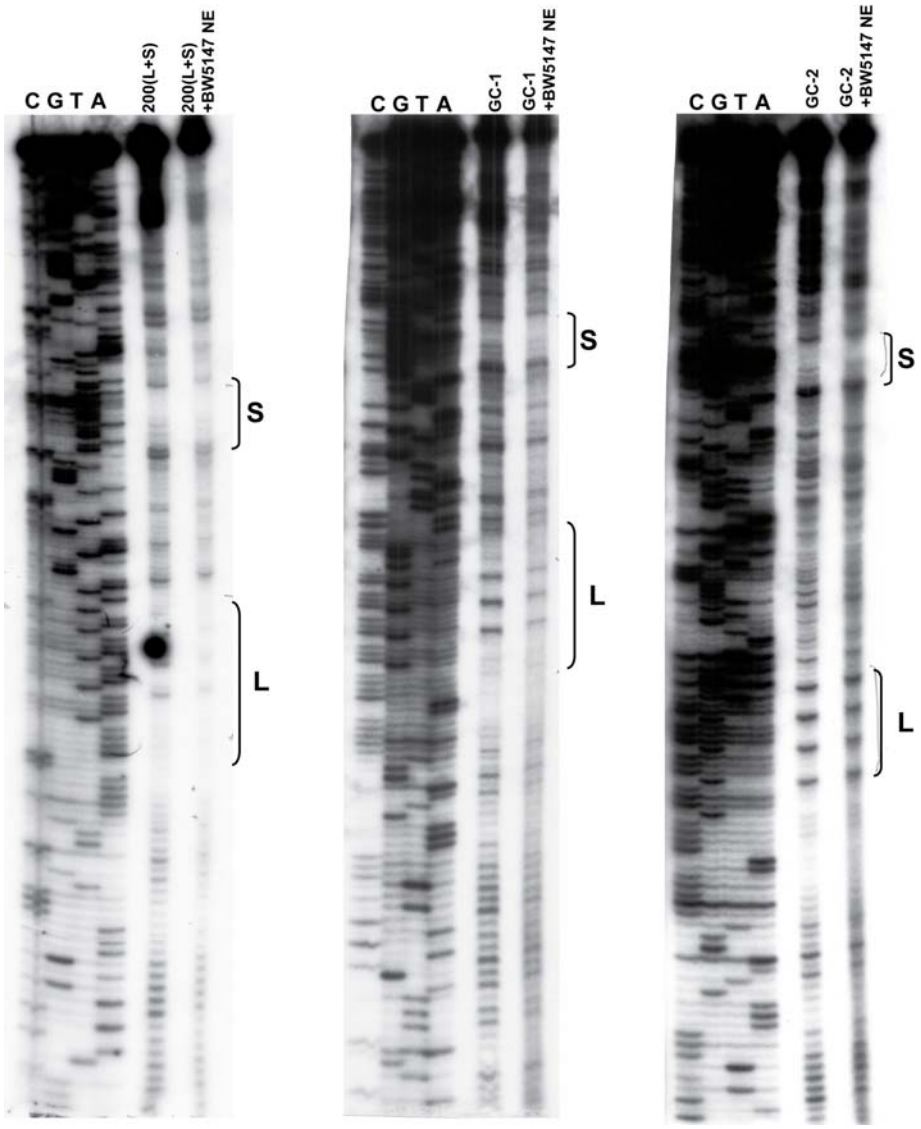


Fig. 12. DNase I footprinting indicates decreased binding of CDP/Cux to L2a.

DNase I footprinting performed with BW5147 nuclear extract, 200(L+S), GC-1, and GC-2 mutated probes. Retarded bands of probe-protein complex and free probes were excised from EMSA (DNase I treated) gels for footprinting analysis. Protected regions are labeled as L and S.

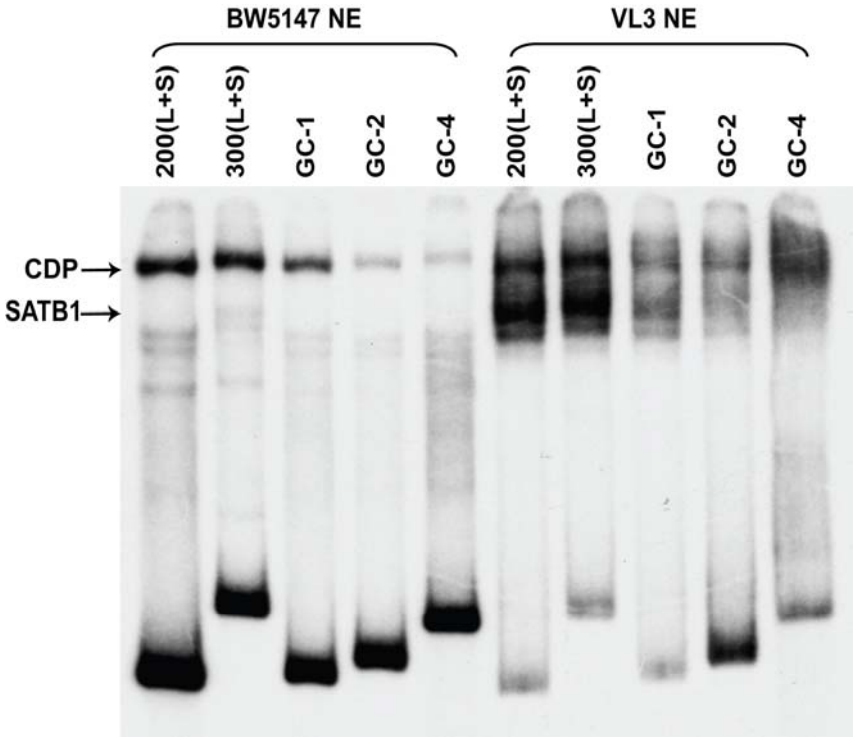


Fig. 13. SATB1 shows a different binding pattern to GC mutant probes.
EMSA performed with BW5147 nuclear extract, VL3 nuclear extract, radiolabeled 200(L+S), 300(L+S) probe and mutated 200(L+S) GC probes. The arrows indicate SATB1 or CDP/Cux complexes.

An EMSA was also performed with mutant RD probes, and similar binding patterns were observed (Fig.14). The SATB1 complex shifted to a higher mass, and it merged into the CDP/Cux complex when the RD-3 probe was used. When the PAL-1 probe (which contains the palindromic 12-mer of L2a) was used with the VL3 nuclear extract, only a single complex was observed. Comparing RD-1 and PAL-1, which are the same size and have similar structures, it seems likely that the presence of a single palindromic insert was responsible for altering the binding patterns of CDP/Cux and SATB1 to the L2a element. This indicates that the palindromic 12-mer in the INTER-LS region is critical for the binding of CDP/Cux and SATB1 to L2a.

Next, we used anti-CDP/Cux and anti-SATB1 antibodies in EMSAs to confirm the identity of the complexes observed in the previous experiments. In Figs. 15 and 16, the EMSAs were performed with the antibodies added to the nuclear extract before the addition of probes. When SATB1 protein was immunodepleted in this fashion, CDP/Cux produced the expected binding pattern (ie, similar to the EMSA performed with BW5147 extract). However, following depletion of CDP/Cux, SATB1 still showed a super-shifted binding pattern with increased sizes of the probes.

One explanation for this result is that the binding of SATB1 to mutant probes causes a conformation change in the DNA. It is known that SATB1 binding sites normally show a propensity to became stably base-unpaired [146]. It is possible that the binding of SATB1 might cause changes in the base-unpairing of the mutant L2a probes, and unpaired DNA may be responsible for the retardation of these SATB1-DNA complexes. Another explanation is that there might be another protein involved in the interaction of the L2a element with CDP/Cux, and SATB1 proteins.

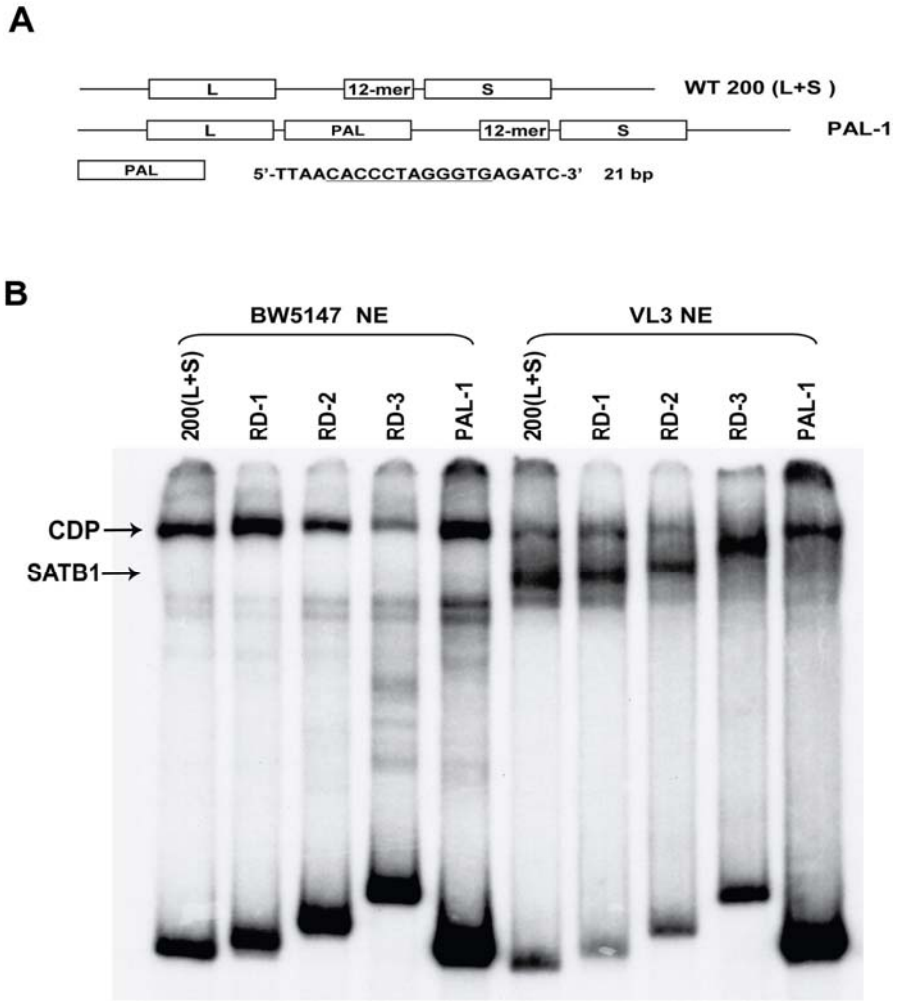


Fig. 14. SATB1 shows a different binding pattern to RD mutant probes.
A. Wild type L2a 200(L+S) and mutant PAL-1 with one PAL insert. **B.** EMSA performed with BW5147 nuclear extract, VL3 nuclear extract, radiolabeled 200(L+S), and mutated RD and PAL-1 probes. The arrows indicate SATB1 or CDP/Cux complexes.

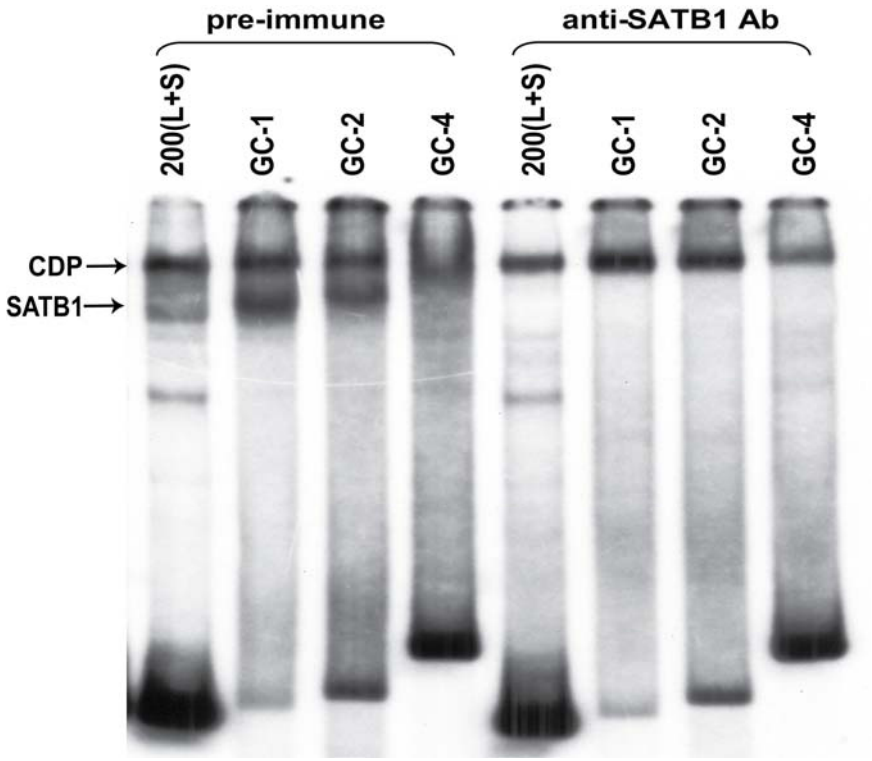


Fig. 15. CDP/Cux binding to L2a is unaltered in the absence of SATB1.
EMSA performed with VL3 nuclear extract, radiolabeled 200(L+S) and mutated GC probes. Anti-SATB1 antibody was added to nuclear extract before incubation with the probes. The arrows indicate SATB1 or CDP/Cux complexes.

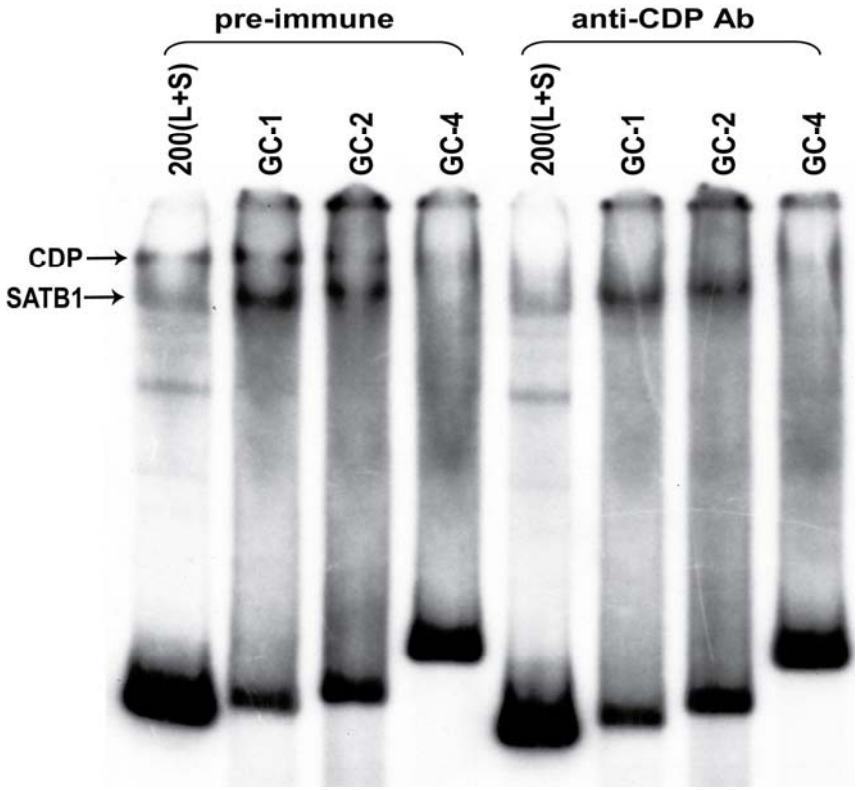


Fig. 16. SATB1-L2a DNA complex is super-shifted in the absence of CDP/Cux.
EMSA performed with VL3 nuclear extract, radiolabeled 200(L+S) and mutated GC probes. Anti-CDP antibody was added to nuclear extract before the probes. The arrows indicate SATB1 or CDP/Cux complexes.

To further investigate the binding properties of SATB1 and CDP/Cux, DNase I footprinting was performed with VL3 nuclear extract as described previously. As shown in Fig.17, protection of both L and S regions were observed in the retarded CDP/Cux complex. As the numbers of inserts in the INTER-LS region were increased, protection of both L and S regions decreased; but the trend of the decrease was not as obvious as that of the footprinting performed with BW5147 nuclear extract.

The footprinting results from the retarded SATB1 complexes demonstrated that there is protection of both the L and the S regions and that the L region seems to be the primary site. When the number of inserts in the INTER-LS region is increased, the protection of the S region had little change, whereas the L region remained strongly protected. This suggested that SATB1 binds to the L site constitutively. Interestingly, the DNase I hypersensitivity of the INTER-LS region upon SATB1 binding changed dramatically. For the wild type 200(L+S) L2a probe, a DNase I hypersensitivity site, consistent with what had been previously described [109], appeared near the palindromic 12-mer. Using the GC-1 and GC-2 probes, significant DNase I hypersensitivity was displayed near the palindromic 12-mer which spread across the entire INTER-LS region. When the GC-4 probe (containing 4 inserts) was used, no hypersensitivity was observed in the INTER-LS region. This suggested that SATB1 binding did not altered the conformation of GC-4 DNA. These results raise the possibility that SATB1 cannot alter DNA conformation over a long distance (GC-4, 88bp insert), but a shorter distance (GC-2, 22bp; GC-2, 44bp) is still deformable.

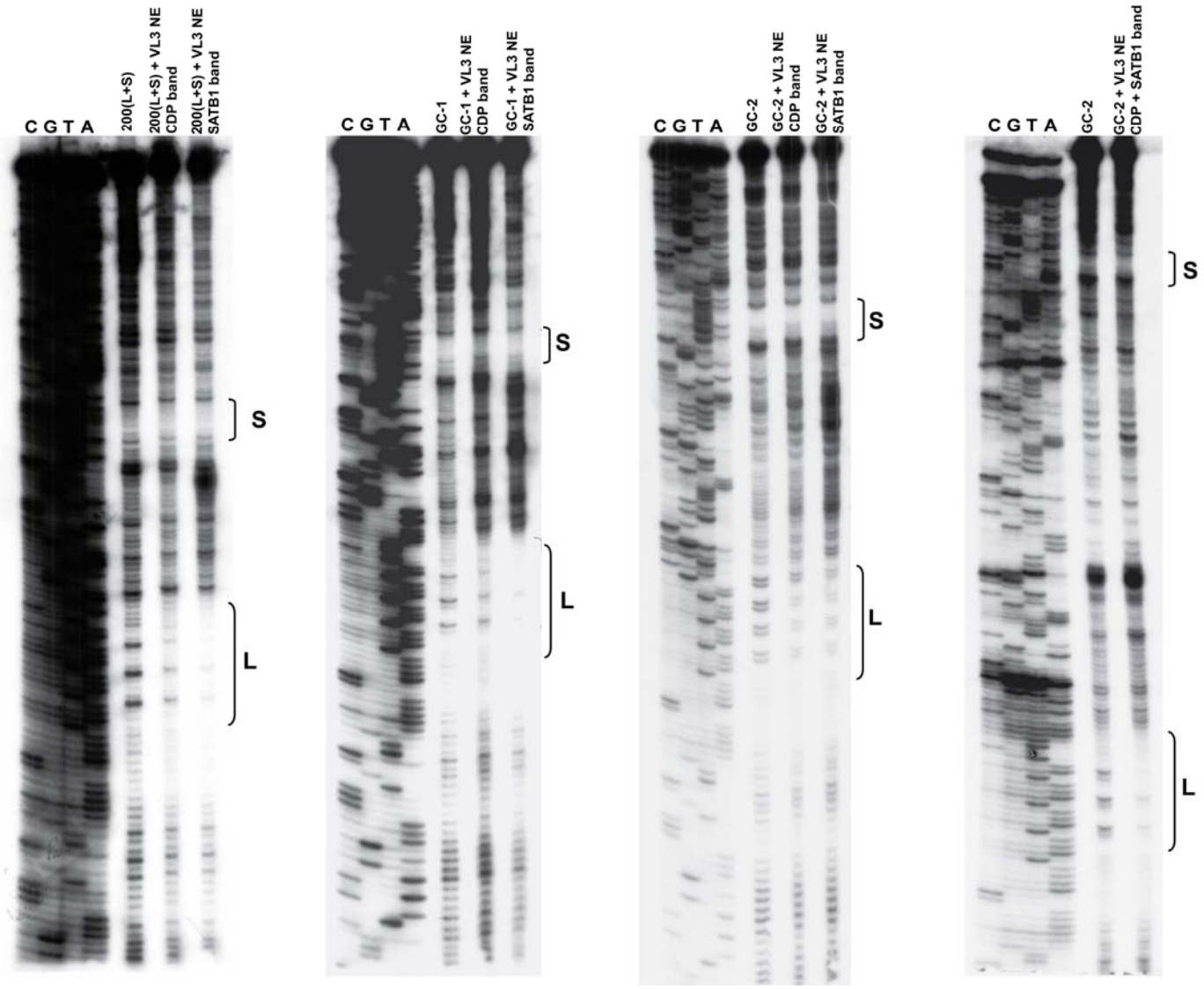


Fig. 17. DNase I footprinting indicates a conformational change(s) is induced within the L2a element upon SATB1 binding.

DNase I Footprinting performed with VL3 nuclear extract, 200(L+S), GC-1, GC-2, and GC-4 mutated probes. Retarded bands of probe-protein complex and free probes were excised from EMSA (DNase I treated) gels for footprinting analysis. Protected regions are labeled as L and S.

3.1.3 L2a mutants have altered nuclear matrix binding

It has been shown that the sequences required for binding of both CDP/Cux and SATB1 contribute to the matrix-binding properties of the L2a element [109]. Thus it was important to know if changing the distance between the L and S regions would alter the matrix-binding properties of L2a. A standard *in vitro* nuclear matrix binding assay [205] was used to exam such effects.

Fig. 18 shows the matrix-binding activities of L2a mutants with nuclear matrix prepared from EL-4 T cells. Compared to the positive control (BrMAR) previously shown [207] to be a strong MAR, the L2a element and its mutants weakly bound the nuclear matrix. When the distance between L and S regions was increased, L2a mutant binding was decreased, and the interaction of GC-4 with the nuclear matrix was significantly weaker than that of wild type L2a. This result suggested that the nuclear matrix binding properties of the L2a element are altered by increasing the distance between the L and S regions.

3.1.4 Multiple unidentified proteins bind to the palidromic 12-mer.

Our previous results demonstrated that a single palindromic insert disrupted the normal binding pattern of CDP/Cux or SATB1 to the L2a element (Fig. 14), suggesting that the palindromic 12-mer may play a role in these interactions. When BW5147 nuclear extract was used to perform EMSA with L2a, a couple of unidentified complexes migrated below the CDP/Cux complex (Fig. 8-11, 13, 14). Since palindromes are frequently sites for protein interactions, it is possible that an unknown protein could account for this pattern.

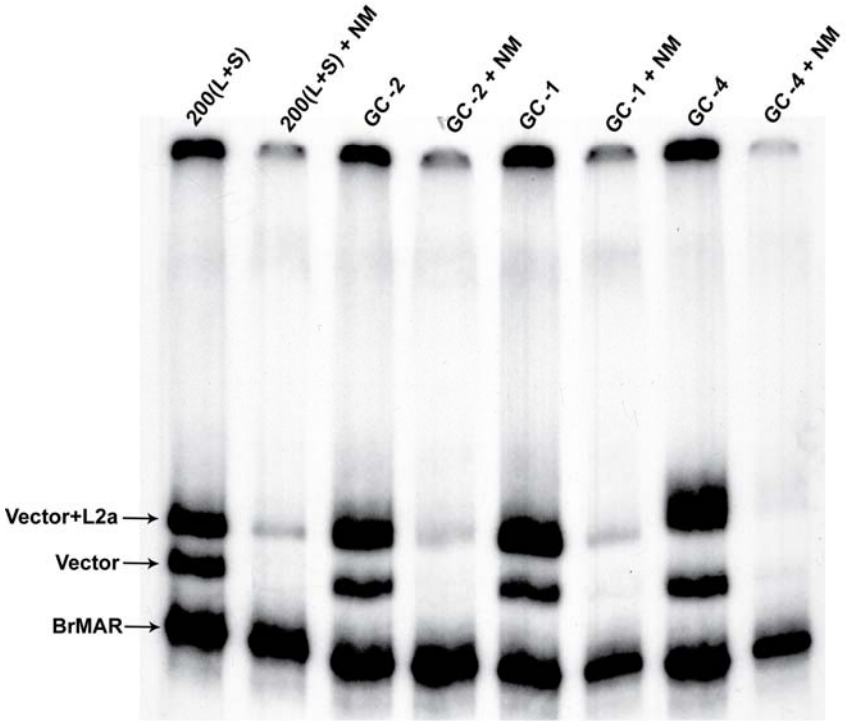


Fig.18. L2a mutants have altered nuclear matrix binding activity.
Linearized, radiolabeled plasmids containing different L2a mutants (GC-1, GC-1, and GC-4) were employed in an in vitro nuclear matrix binding assay. Linearized, radiolabeled empty vector (negative control) and BrMAR (an established MAR, positive control) were quantitated and added to each reaction. NM, nuclear matrix prepared from EL-4 T cells.

A mutant probe, Nco11, with two point mutations disrupting the palindromic 12-mer, was constructed and employed in EMSA (Fig. 19A). The retarded bands A and B of putative unidentified proteins disappeared were eliminated (Fig. 19B), suggesting that the 12-mer may be their binding site. Interestingly, the mutant probe favored the binding of SATB1 to the L2a element when VL3 nuclear extract was used, further indicating that the 12-mer may crucial to these interactions.

Three additional constructs were made by inserting the palindromic sequence (PAL) into the middle of the 12-mer (Fig. 20A). Mutants PL-1, PL-2, and PL-3 have one, two, and three PAL inserts, respectively. As shown in Fig. 20B, the intensities of bands A and B increased when probes with additional 12-mers were used. These results suggest that probes with additional 12-mers bind stronger.

Competition experiments were carried out using annealed palindromic oligos (PAL) and random sequence oligos (RAD) as competitors in EMSAs performed with BW5147 nuclear extract, 200(L+S), and PL-3 probes (Fig. 21). The palindromic oligos were found to compete with 200(L+S) and PL-3 probes. They specifically reduced, and even eliminated, the formation of complexes A and B, whereas the random oligos had no effect. These results suggest that the palindromic 12-mer may be the binding site of unidentified proteins.

3.1.5 Identification of the 12-mer binding proteins

The two retarded L2a palindromic binding complexes (A and B) were estimated from the EMSAs to run at 50-80 KDa. They were present in EMSAs performed with the CD8 and SATB1-negative BW5147 nuclear extract. DNA-affinity chromatography was used to purify the proteins that specifically interact with palindromic 12-mer sequence.

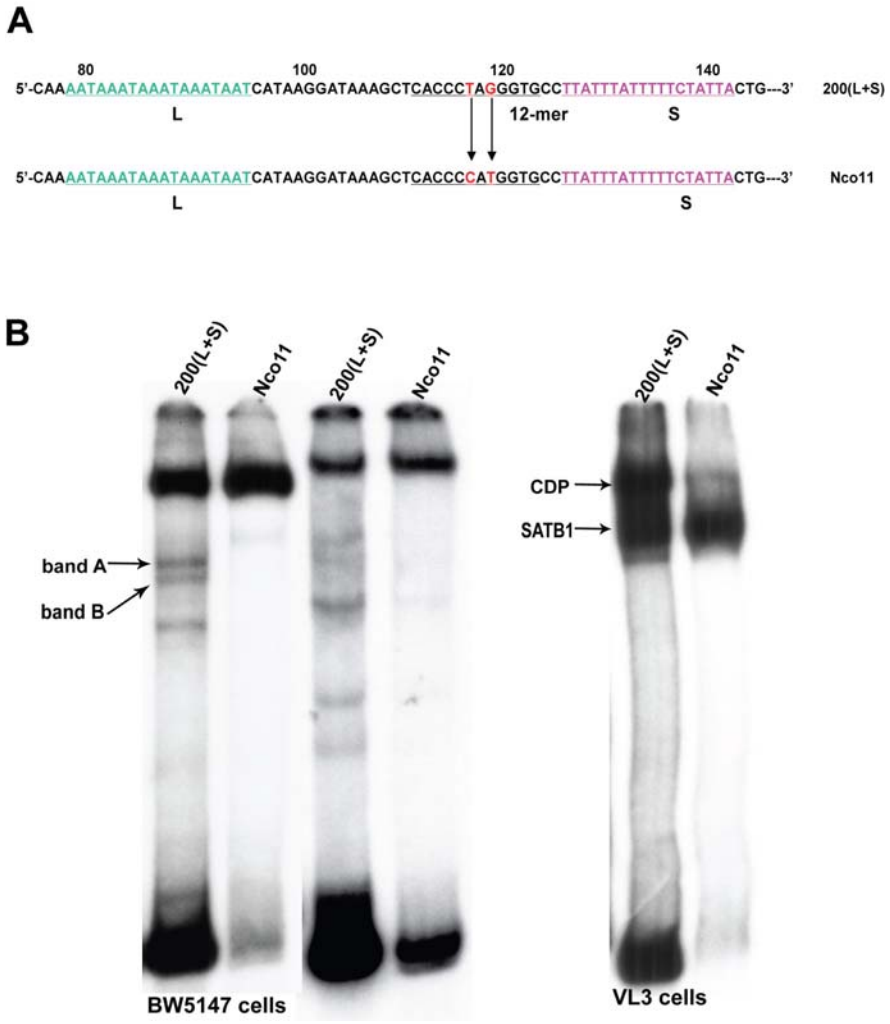


Fig. 19. Mutations of a palindromic 12-mer within L2a abolish unidentified protein-DNA complexes formed in BW5147 nuclear extract.
A. Schematic of L2a showing 12-mer and two point mutations (red) within (Nco11 probe). **B**. Left, two independent EMSAs performed with BW5147 nuclear extract, 200(L+S), and Nco11 probes. Complexes A and B are indicated by arrows. Right, mutations in palindromic 12-mer appear to favor the binding of SATB1 in VL3 nuclear extract.

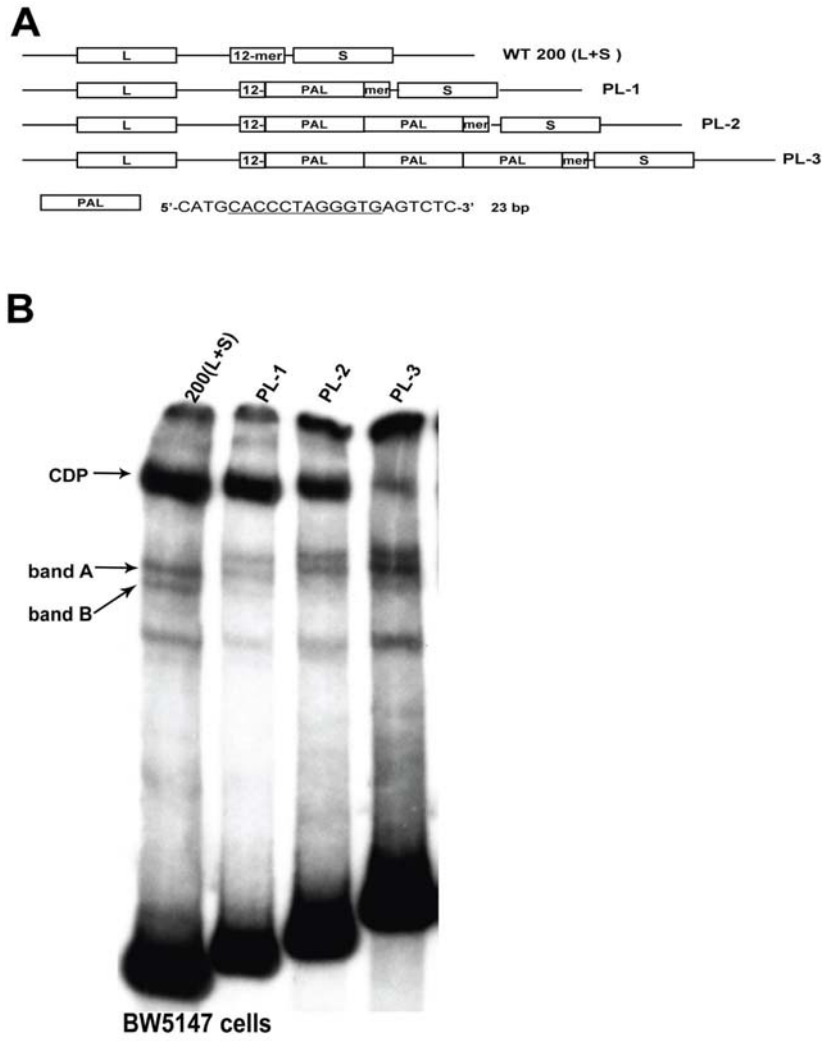


Fig. 20. Insertion of additional 12-mers into L2a probes lead to stronger bands A and B.

A. Wild type L2a and mutant PL probes with PAL inserts. PL-1, one palindromic insert; PL-2, two PAL inserts; PL-3, three PAL inserts. **B.** EMSA performed with BW5147 nuclear extract, radiolabeled 200(L+S) probe and mutated PL probes.

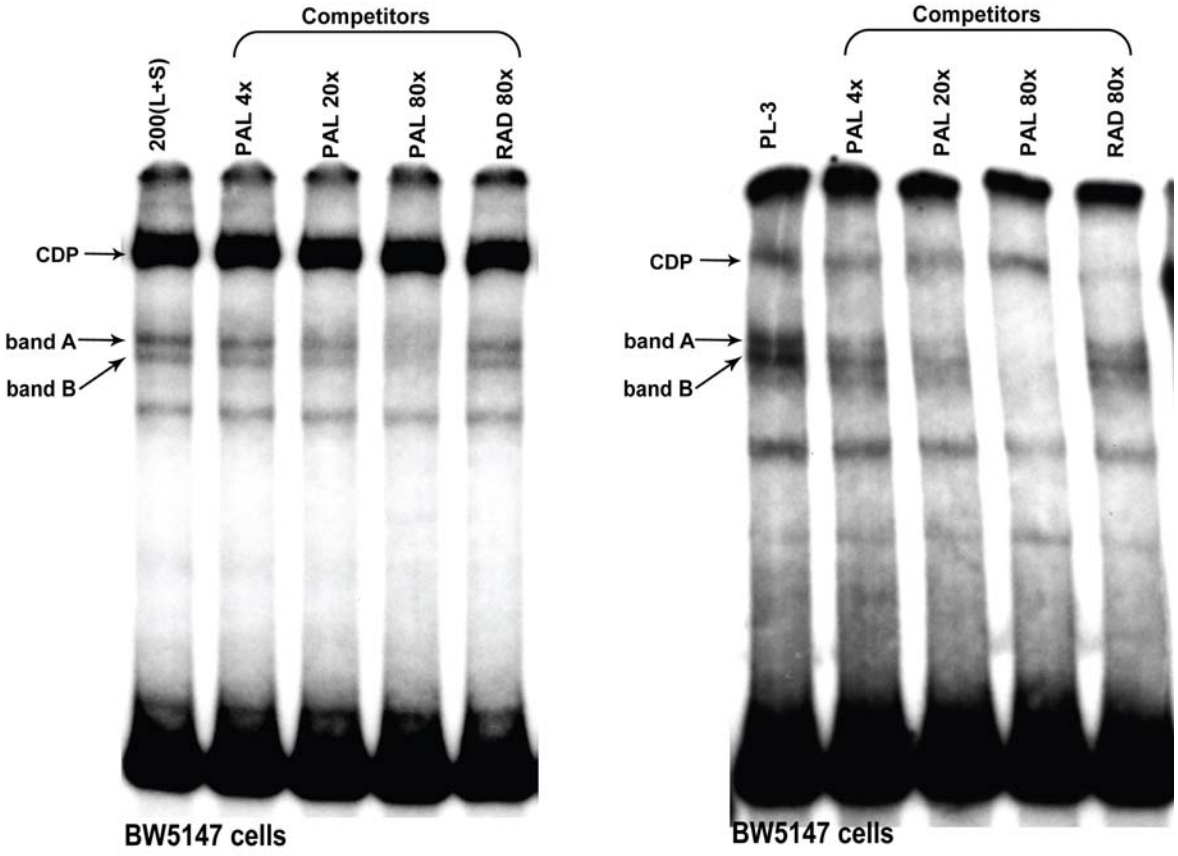


Fig. 21. Palindromic oligos specifically compete with 200(L+S) and PL-3 probes for formation of bands A and B.
Competition EMSA performed with BW5147 nuclear extract, radiolabeled 200(L+S) probe (left), PL-3 (right) and unlabeled ligated palindromic or random oligo competitors.
PAL oligo: 5-TTAACACCCTAGGGTGAGATC-3';
RAD oligo: 5'-TTAAAGGACACTAGATCTGTAC-3'

To determine the DNA ligand for the affinity purification, three truncations of mutant probe PL-3, which has three palindromic inserts and exhibited the strongest binding in EMSA, were constructed by PCR. Probe L+P3 consists of the L region and three PAL sequences; probe S+P3 consists of the S region and three PAL sequences; probe P3 has only the three PAL inserts (Fig. 22A). As shown in Fig. 22B, the P3 probe bound weakly to proteins found in complexes A and B. Probe S+P3 produced stronger A and B bands than P3, whereas probe L+P3 failed to form either complex. This result suggests that both the S region and palindromic sequence are required for formation of bands A and B, and probe S+P3 is the best DNA sequence for affinity purification because it produces strong A+B bands and abolishes the binding of CDP/Cux.

Ligated PCR-amplified S+P3 probe was coupled to CNBr-activated Sepharose 4B beads to make the DNA affinity column. Nuclear extract prepared from BW5147 cells was diluted and passed through the uncoupled column first to trap non-specific protein binding, and then twice through the S+P3 column. The proteins that bound to the S+P3 column were eluted with buffer at higher salt concentration. Eluted samples were applied to EMSA to check the efficiency of purification (Fig. 23A). These experiments indicated that CDP/Cux was completely depleted and the purified fraction retained binding ability to the 12-mer. Coomassie stained SDS PAGE revealed two abundant bands (Fig. 23B), and both of them were cut out of gel and sent to Core-facility (UT-Austin) for Mass Spectrometry (MS) analysis.

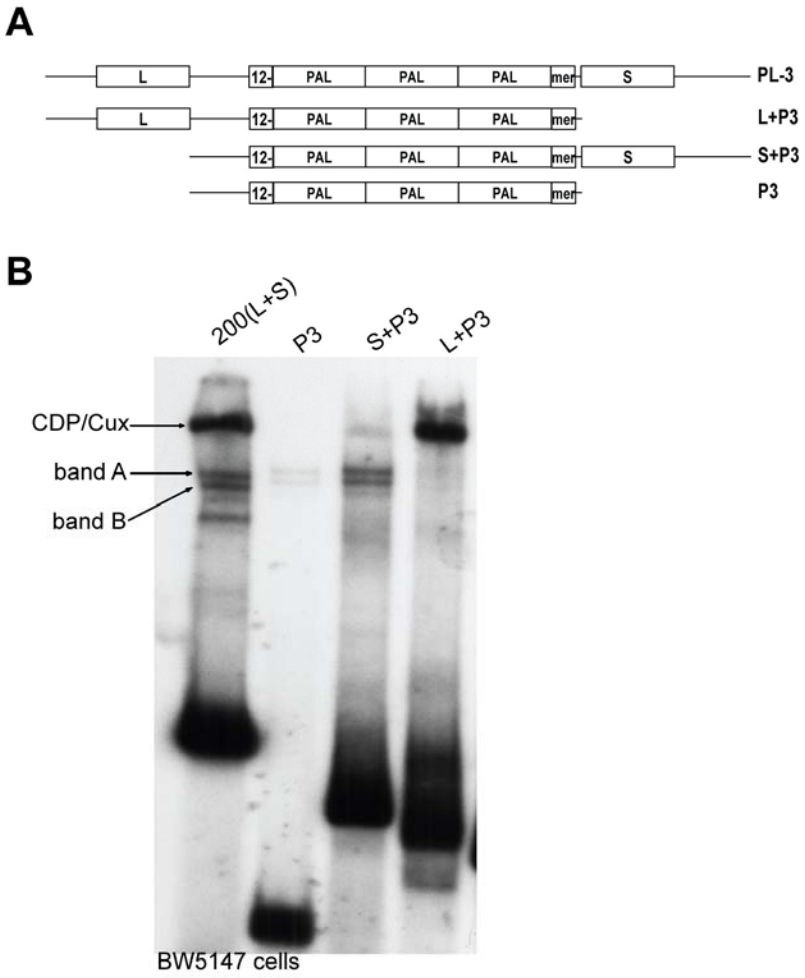


Fig. 22. Determination of Probe S+P3 as the optimal DNA sequences for DNA-affinity purification of palindrome binding proteins.
A. Mutant probes P3, S+P3, and L+P3 were created by PCR based on PL-3 probe. **B.** EMSA performed with BW5147 nuclear extract, 200(L+S) and mutant probes. Both S region and palindromic sequence are required for formation of bands A and B.

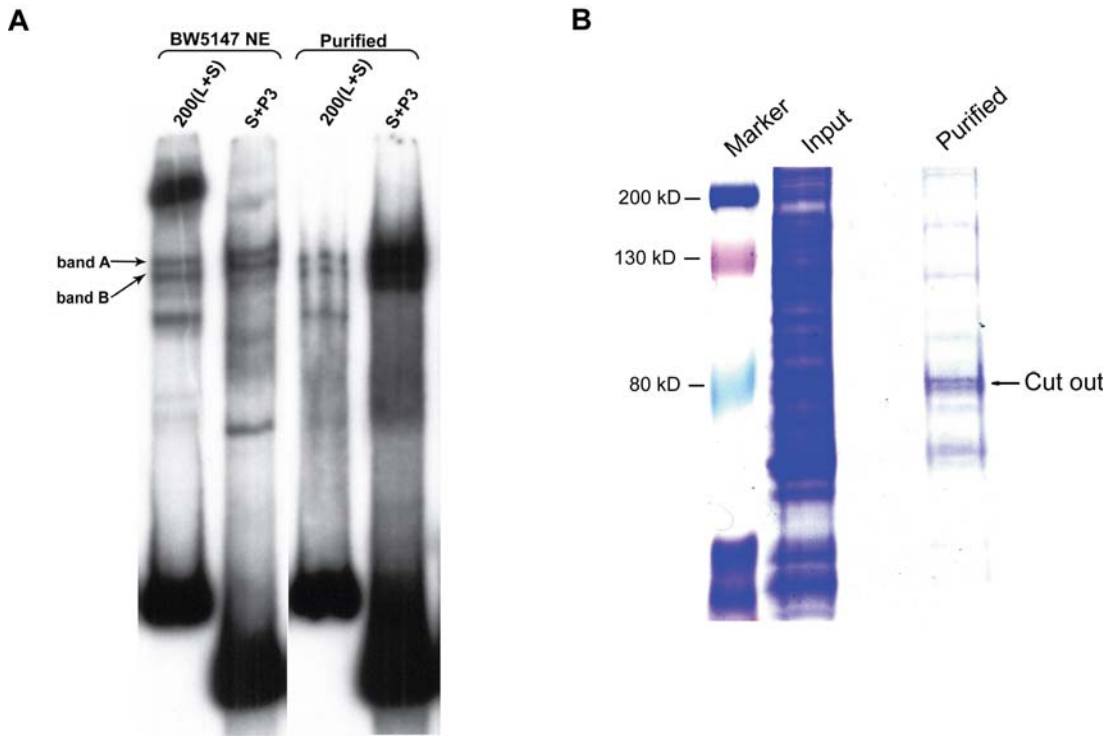
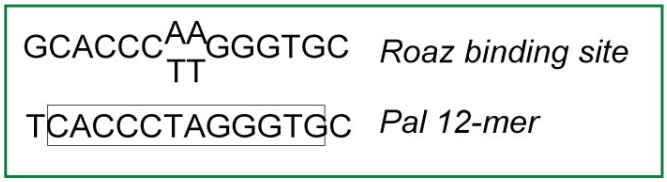


Fig. 23. Identification of two palindromic binding proteins by affinity purification.
A. EMSA performed with purified 12-mer binding proteins, 200(L+S), and S+P3 probes. CDP/Cux was completely depleted and the purified fraction retained binding ability to S+P3. **B.** Coomassie stained SDS PAGE revealed two intensified bands, which were excised from the gel and analyzed by Mass Spectrometry (MS).

Based on the mouse genome database, MS revealed two candidates with high scores. One of them is the protein pigpen, which is a nuclear protein modulating endothelial cell differentiation [208]. Pigpen exhibits a complex structure, including a transcriptional activation (TA) domain, a zinc finger-like hairpin loop motif, a consensus tyrosine phosphorylation site, and two consensus RNA binding motifs [208]. The other protein identified by MS is an unnamed putative protein (gi/26334035). A search for conserved domains suggested it has a zinc finger structure and an actin depolymerisation factor (ADF)-like domain.

Another candidate for the 12-mer binding protein was discovered after the analysis of the L2a sequence using a transcription binding factor data base (www.genomatix.de). The rat Olf-1/EBF associated zinc finger protein, ROAZ, binds to an inverted perfect or imperfect repeats of GCACCC separated by 2 bp [209]; this is almost identical to the 12-mer in the L2a element. Roaz is a C_2H_2 zinc finger protein that plays a role in the regulation of olfactory neuronal differentiation [209]. This protein contains both a protein interaction and a DNA binding domain along with 29 C_2H_2 zinc fingers. This protein family also includes OAZ (Olf-1/EBF associated zinc finger, human) [210] and Ebfaz/Evi3 (early B-cell factor associated zinc finger protein/ecotropic viral integration site 3, mouse) [211]. Ebfaz/Evi3 has been shown to be essential for normal mouse B cell development and a frequently targeted locus for murine retroviral-mediated leukemogenesis [211].



To determine if the OAZ family proteins interact with L2a element and the palindromic 12-mer, OAZ and Ebfaz cDNA constructs were obtained from the labs that identified them. Proteins were synthesized using an *in vitro* translation kit (Promega), and products were employed in EMSA using L2a probes. Unfortunately, *in vitro* translation failed to produce the detectable amounts of OAZ or Ebfaz proteins, probably, because of the complexity their structures (29 zinc-fingers). Further analyses are required to determine the identity of any of the putative 12-mer binding proteins.

3.1.6 Cell transfection assays implicate the L2a element as a silencer

To gather direct evidence for consequential CD8 transactivation via the L2a element, a series of luciferase vectors based on pGL3-basic (Promega) were constructed for use in transfection studies (Fig. 24A). Initial efforts to employ them in transient co-transfections of T cell lines, including BW5147 cells, VL3 cells and 1200M cells, gave low activities and inconsistent results (data not shown). Other efforts to introduce SATB1 into SATB1-null cells or to knock down SATB1 expression by siRNA were not successful (data not shown). Cell lines other than the above T cell lines were also employed in transfection assays using these L2a constructs. The embryonic kidney epithelial cell line, 293T, yielded consistent but unexpected results. As shown in Fig. 24B, the construct L2aD-Luc, which lacks the L2a element, increased luciferase activity ~2-fold as compared with the L2a-containing constructs, indicating a possible negative regulatory role for L2a.

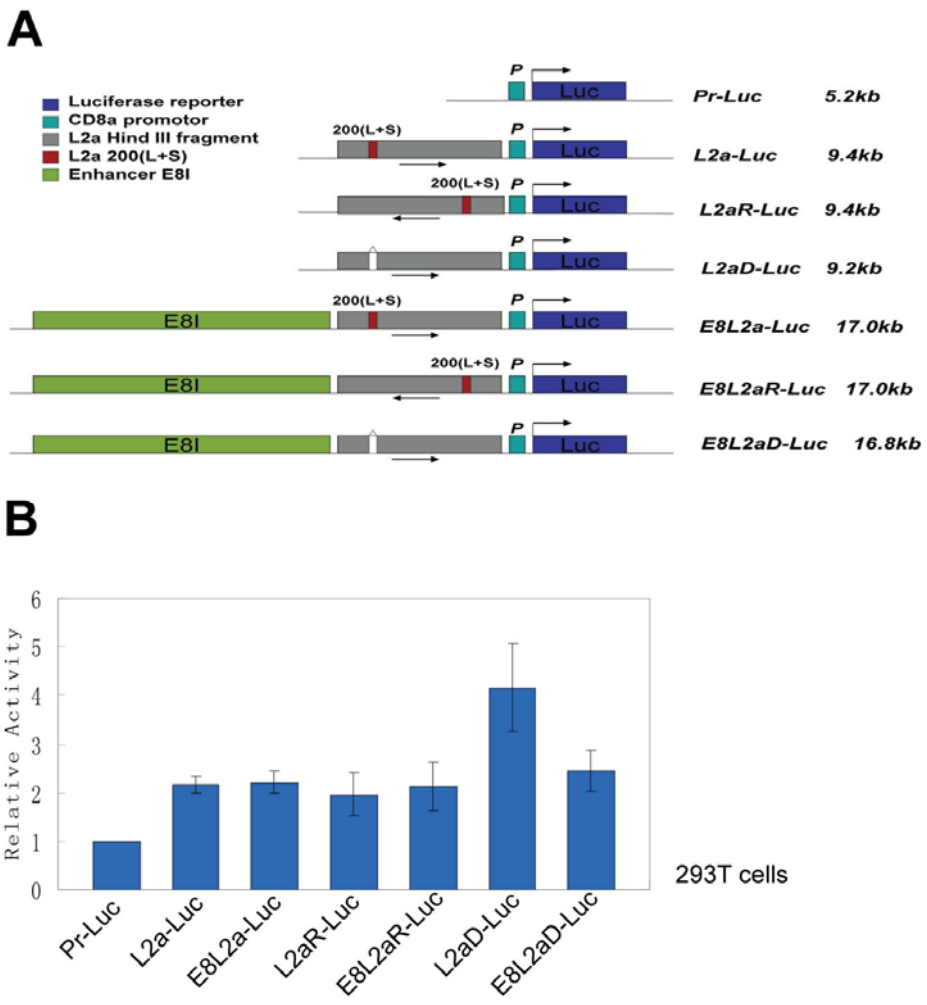


Fig. 24. Transfections of 293T cells suggest a negative regulatory role for L2a on the E8I enhancer.

A. L2a luciferase vector series constructed for the transfection studies. Components of the constructs are highlighted in different colors. **B.** 293T cells were transfected with L2a luciferase constructs and *Renilla* luciferase vector. Cell lysates were measured for dual luciferase activities. Relative activities were normalized with *Renilla* activities.

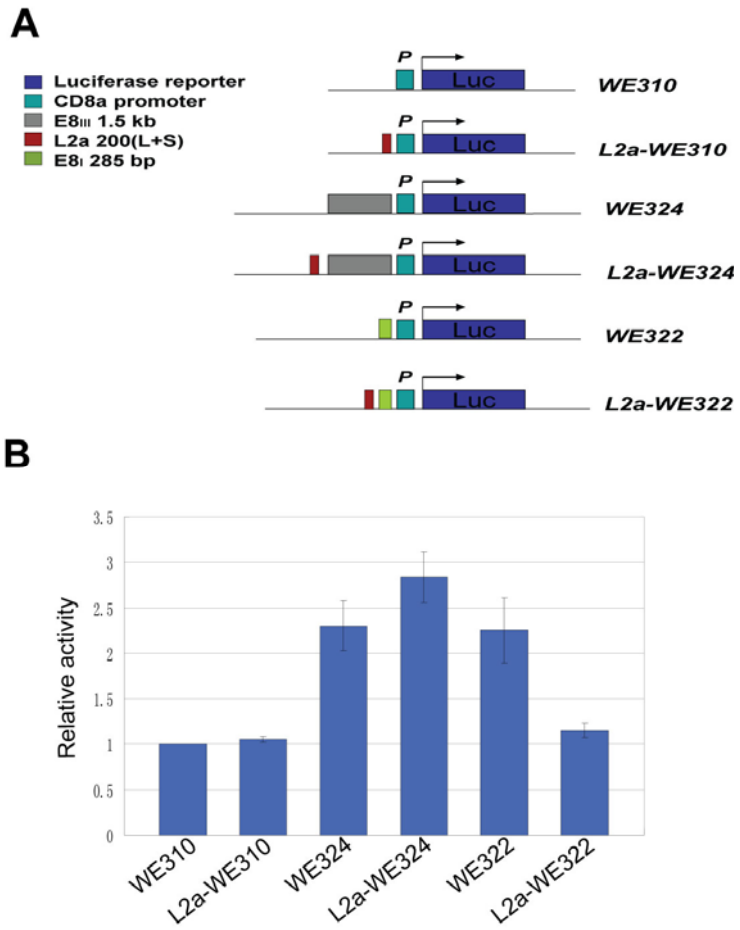


Fig. 25. Transfections of 1200M cells indicate a negative regulatory function of L2a on E8III enhancer.
A. L2a-WE luciferase vector series was created based on WE310, 324 and 322 vectors. Components of the constructs are highlighted in different color. **B.** 1200M T cells were transfected with L2a-WE luciferase constructs and *Renilla* luciferase vector. Cell lysates were measured by dual luciferase assays. Relative activities were normalized with *Renilla* activities.

Feik and coworkers [64] identified a 285 bp core enhancer within the E8III enhancer using luciferase assays. This was one of the few successful results obtained from transfection studies on CD8 *cis*-acting elements. We decided to test L2a in the context of the E8III enhancer using as starting materials the "WE" constructs discussed in that report [64] and kindly provided by Dr. Wilfried Ellmeier. A 220 bp L2a fragment was cloned upstream of the E8III fragment of each of the WE plasmids, and structures of all L2a-WE constructs are shown in Fig.25A. 1200M T cells were transfected with L2a-WE constructs, and results are shown in Fig. 25B. L2a-WE322, which contains the L2a element and the 285 bp E8III core, gave a ~2-fold decrease in relative activity compared to other constructs bearing E8III sequences. The activity of L2a-WE324 was not affected, perhaps because the distance between L2a and the core enhancer is longer than that of L2a-WE322. These results suggest a possible negative role for L2a when in *cis* with E8III.

3.1.7 EMSAs revealed two more SATB1binding regions in DH cluster II

A knockout study suggested that targeted deletion of a 3.4kb genomic region (DH cluster CII-1 and CII-2) containing the L2a element leads to variegated CD8 expression in thymocytes [59], even though DH cluster II was inactive as a transgene [52]. Thus, it seemed possible that multiple *cis*-acting elements (besides L2a) are present in this region. Seventeen probes (each ~250 bp) spanning the entire 4.3 kb HindIII/HindIII cluster II region were constructed by PCR (Fig. 26). EMSAs performed with VL3 cell nuclear extract identified two additional SATB1 and CDP/Cux binding sites present in DH cluster II (Fig. 26). L10 and L12 are close to each other and are separated by L11, a weak binding site for both proteins. Multiple CDP/Cux binding sites were observed in the EMSA above, and assays preformed with BW5147 nuclear

extract yielded similar results (Fig. 27). Antibody super-shifts confirmed that the complexes produced by L10 and L12 contained SATB1 and CDP/Cux (Fig. 28).

The newly identified STAB1 binding sites are proximal to the second DH site in cluster II (CII-2), and the L2a element is near the DH CII-1 site. Another SATB1 binding site, identified in the E8III enhancer (see below) is located close to the third DH site of cluster IV, CIV-3. This overlap between SATB1 binding sites and DNase I hypersensitive sites supports the previous notion [159] that SATB1 is important for remodeling chromatin. The presence of SATB1 binding sites may provide useful clues to identify new DNA *cis*-acting elements.

3.1.8 An additional SATB1 binding site within the E8III enhancer

Recent studies suggest that the CD8 enhancer E8III may function in the proposed coreceptor reversal process during positive selection of thymocytes [65]. Unpublished results from our laboratory (H. Nie, personal communication) suggested that SATB1 is required for coreceptor reversal in CD8SP cell development. This was based on the observation that DP thymocytes isolated from SATB1-null mice and then stimulated in culture with PMA+I initially terminate CD8 transcription to become $CD4^{+}8^{-}$ thymocytes, but never re-initiate CD8 transcription. Since both the SATB1 protein and E8III enhancer appear to be important for the co-receptor reversal process, additional efforts were made to reveal additional connection between these factors.

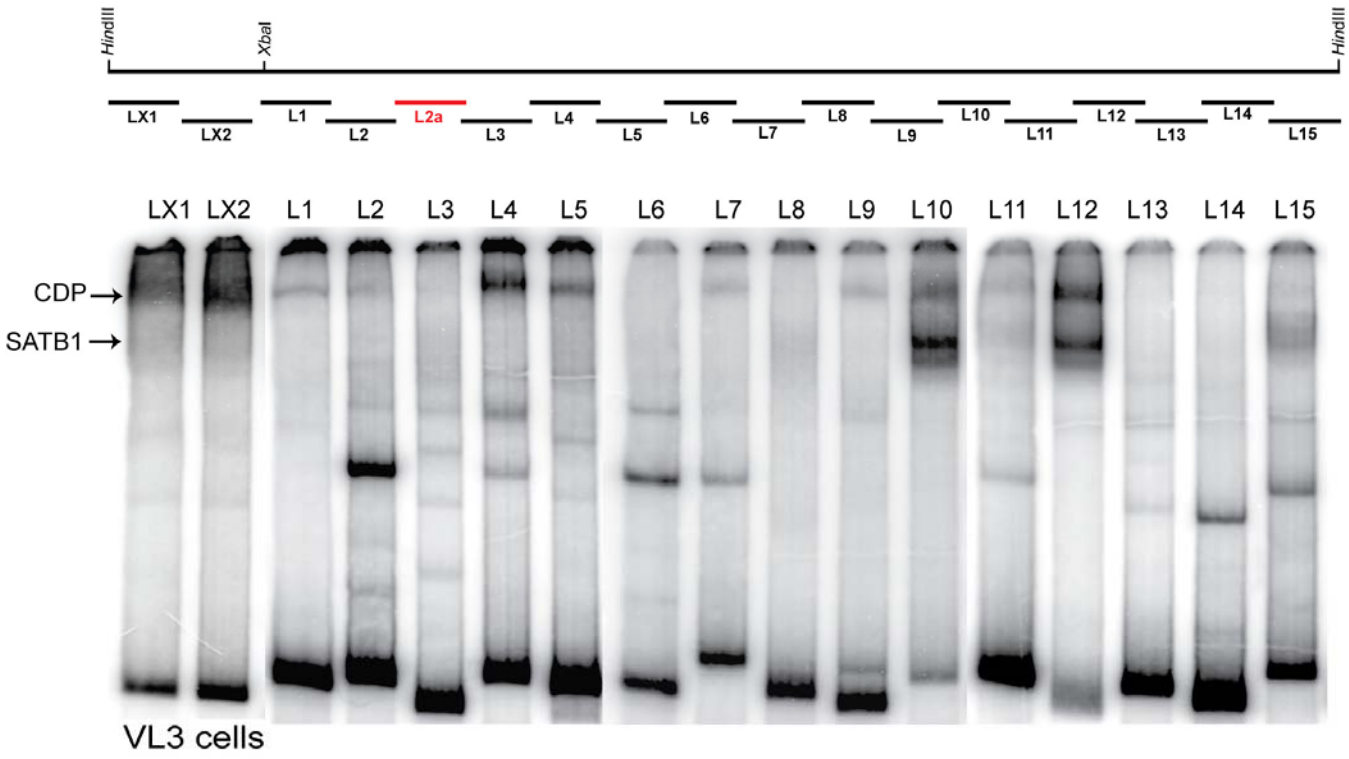


Fig. 26. Identification of two additional SATB1 binding regions within DH cluster II. EMSA performed with VL3 cell nuclear extract and radiolabeled DH cluster II probes covering a 4.3 kb HindIII/HindIII region. The arrows indicate SATB1 or CDP/Cux complexes

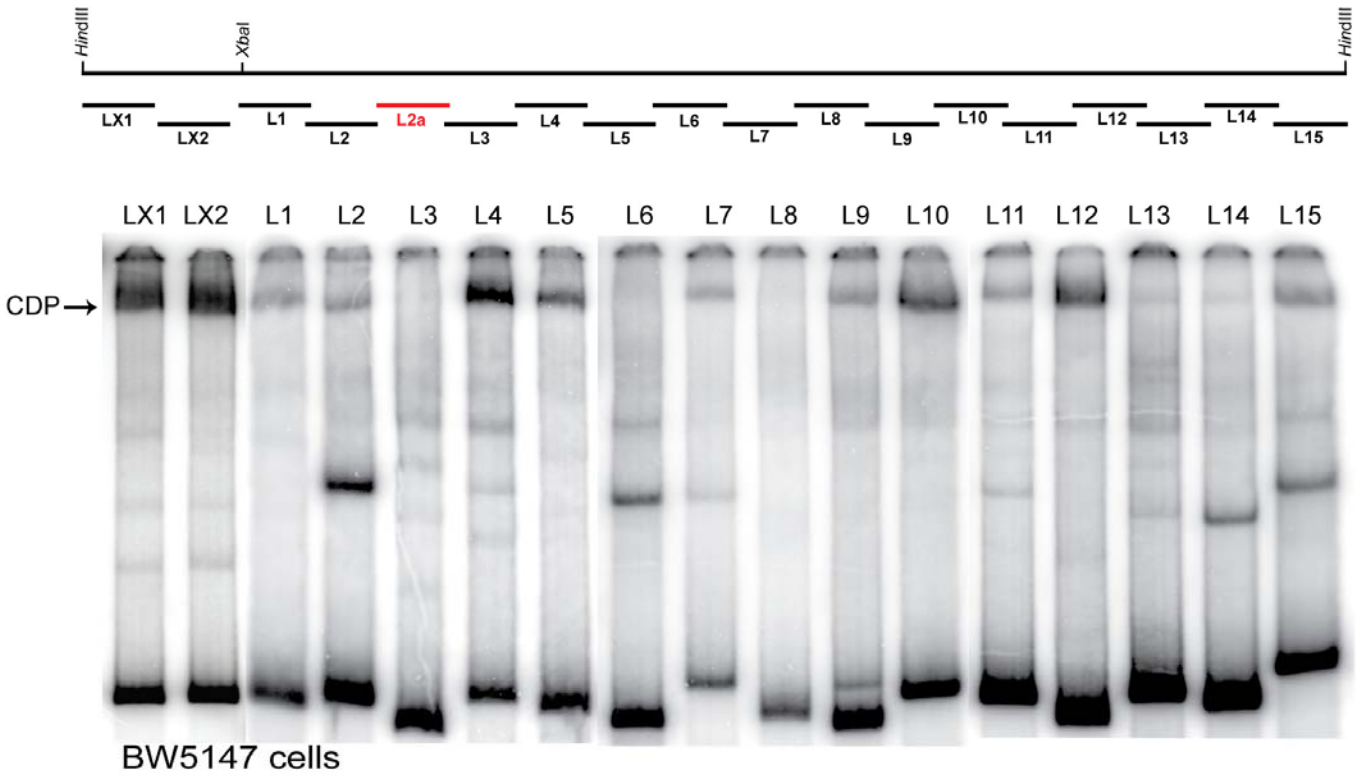


Fig. 27. Multiple CDP/Cux binding sites are present within DH cluster II.
EMSA performed with BW5147 cell nuclear extract and radiolabeled DH cluster II probes covering a 4.3 kb HindIII/HindIII region. The arrow indicates CDP/Cux complexes.

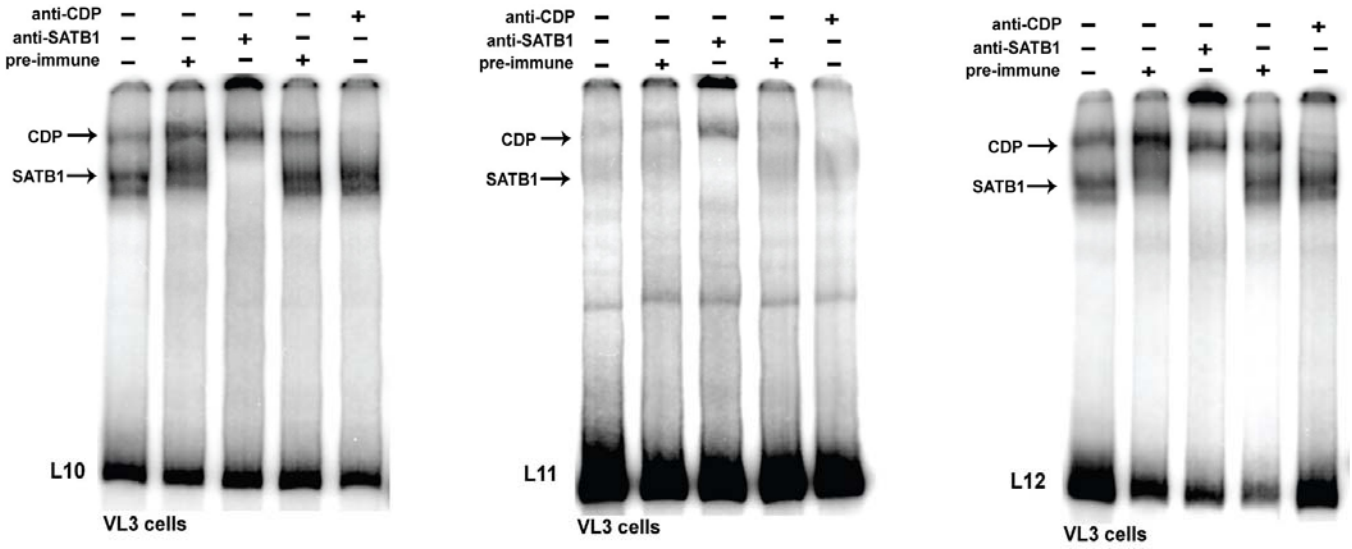


Fig. 28. Antibody supershift/EMSAs confirmation of additional (non-L2a) binding sites for SATB1 and CDP/Cux within DH cluster II.
Supershift/EMSAs were performed with VL3 cell nuclear extract, radiolabeled L10, L11, and L12 probes. Anti-SATB1 or anti-CDP antibodies and pre-immune serum were added to the reaction before the addition of radiolabeled probes. The arrows indicate SATB1 or CDP/Cux complexes.

EMSAs were used to determine potential SATB1-binding regions in the E8III enhancer. A 4 kb EcoRI/BamHI sub-region of E8III has been shown to be active in transgenic assays [57], and further studies using a 1.5 kb SacI/BamHI fragment demonstrated that E8III is involved in coreceptor reversal [65]. Ten probes of approximately 200 bp that span ~2.2 kb across the SacI/BamHI fragment were constructed (Fig. 29). Restriction sites for EcoRI and BamHI were appended to the 5' ends of the PCR primers, and amplified probes were cut by both enzymes and purified for [^{32}P] end labeling.

Nuclear extracts of Jurkat T cells, which express both SATB1 and CDP/Cux proteins, were used in EMSAs to determine if SATB1 can interact with any of these E8III probes (Fig. 29). P12, which is located at the 5' end of 1.5 kb region, produced two retarded bands. Antibody super-shift/EMSAs confirmed that the lower and upper bands are SATB1 and CDP/Cux, respectively (Fig. 31A). Another weaker SATB1-associated region was found in probe P5. Binding of CDP/Cux to P5 was almost undetectable (Fig. 31B). The retarded band observed with probe P2 (which contains the 285 bp E8III core enhancer) was also confirmed as a CDP/Cux complex (Fig. 31C).

The above experimented were repeated with nuclear extract prepared from VL3 T cells. Interestingly, P12 and P5 probes failed to form SATB1 complexes (Fig. 30). However, the CDP/Cux bands remained intact with P12 as well as with P2 and P7. One possible reason responsible for these different binding patterns is that the interaction between E8III sites and SATB1 may be cell-type dependent. Jurkat cells are $CD4^+CD8^-$, and VL3 cells are $CD4^-CD8^+$. Perhaps SATB1 may be differentially post-translationally modified to function uniquely in specific cell types. If this is the case, SATB1 could interact with the E8III enhancer in CD4SP T cells to render it active (or inactive) to function at this stage. The enhancer might become inactive (or active) in CD8SP cells,

because modified SATB1 fails to bind to it. As a further extension to this hypothesis, SATB1 might assist the E8III enhancer in driving the coreceptor reversal process during the $CD4^{+}CD8^{low}$ stage. Modification of SATB1 would result in lost activity once the transition to the CD8SP stage is initiated.

CDP/Cux formed a complex with probe P2, a 285 bp AvaI/BamHI fragment, which was previously identified as the core region of the E8III enhancer in luciferase assays [64]. When we replaced the 285 bp core with the SATB1-binding P12 and P5 fragments identified above, no significant change in relative luciferase activities were observed (data not shown). Our transient transfection assays may not have been capable of identifying the function of SATB1, which has been implicated in regulating tissue-specific gene expression by organizing higher order chromatin structure [159].

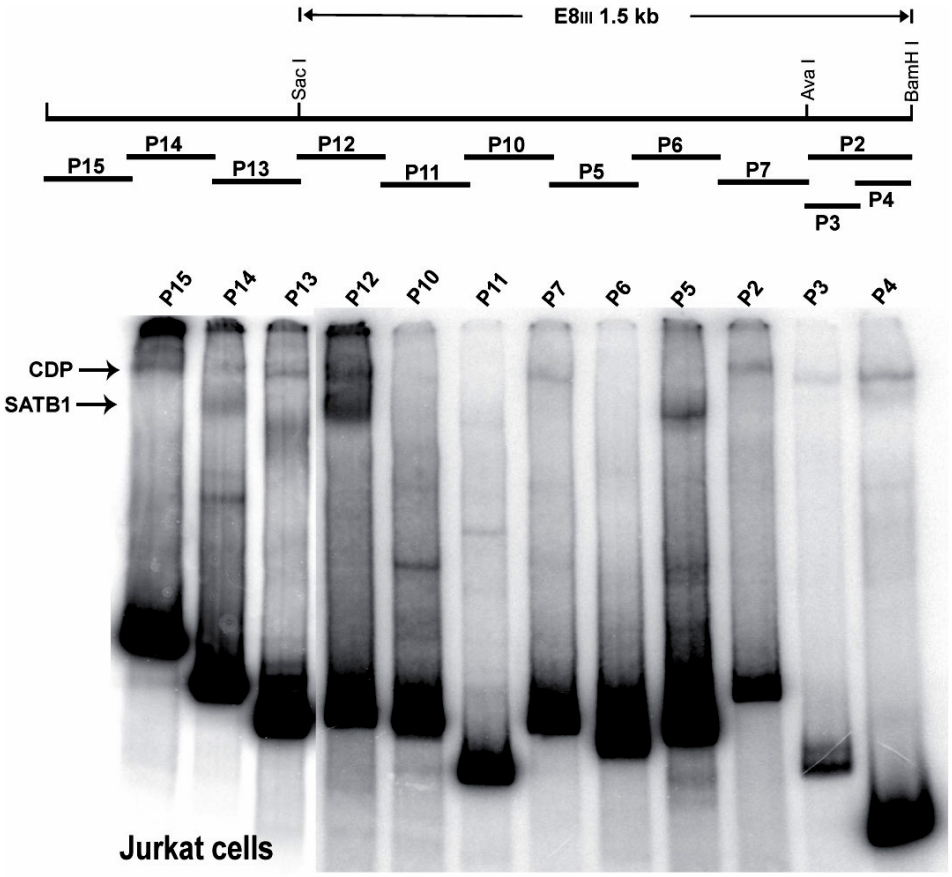


Fig. 29. Additional SATB1 binding sites are present within the E8III enhancer.
EMSA performed with Jurkat cell nuclear extract and radiolabeled E8III probes covering a 2.2 kb region. The order of probes loaded in wells is not same as that shown in schematic map above. The arrows indicate SATB1 or CDP/Cux complexes.

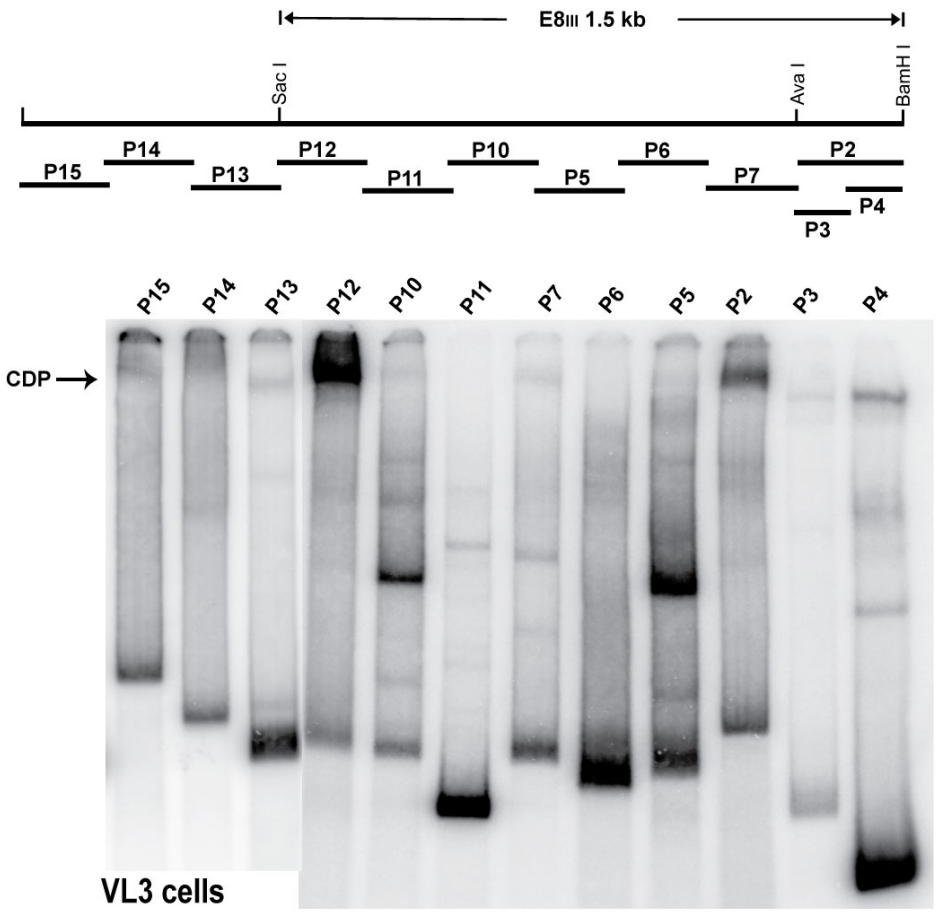


Fig. 30. E8III probes that bind SATB1 in Jurkat nuclear extract do not form SATB1 complexes in VL3 nuclear extract.
EMSA performed with VL3 cell nuclear extract and radiolabeled E8III probes covering a 2.2 kb region. The order of probes loaded in wells is not same as that shown in schematic map above. P12 and P5 probes do not form SATB1 complex in VL3 nuclear extract. The arrow indicates CDP/Cux complexes.

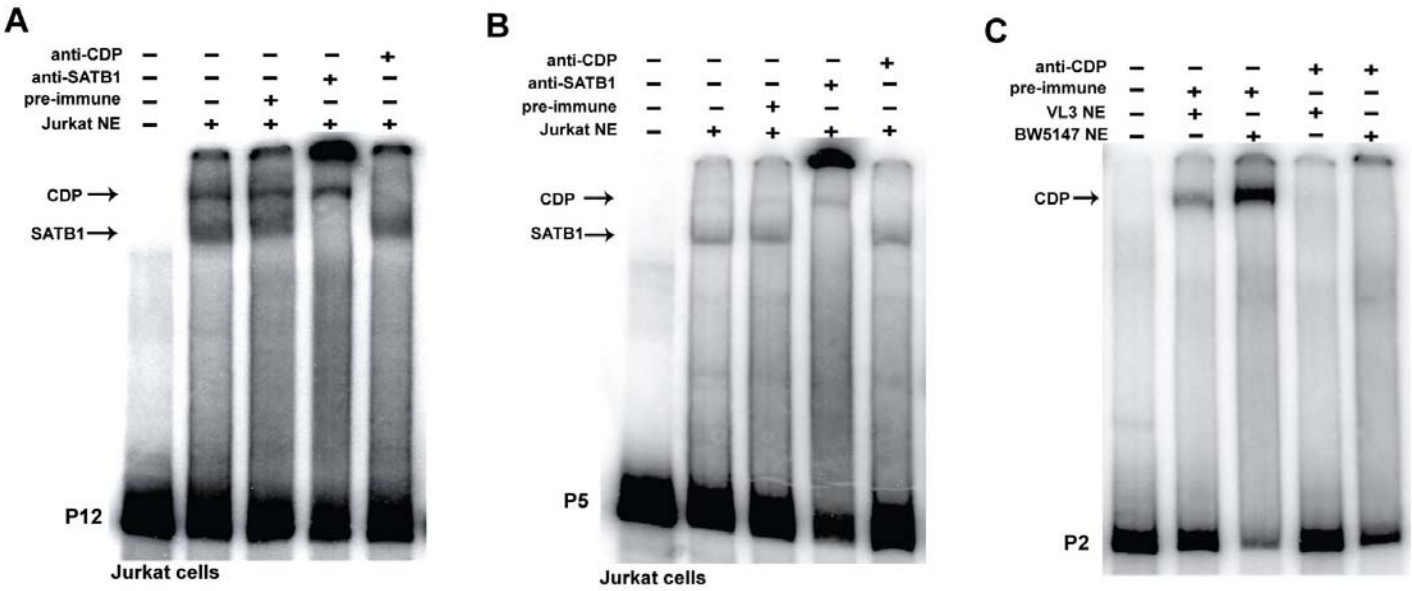


Fig. 31. Supershift EMSAs confirmed additional binding sites for SATB1 and CDP/Cux within the E8III enhancer.
A and **B.** EMSAs performed with Jurkat cell nuclear extract, radiolabeled E8III P12 and P5 probes. Anti-SATB1 or anti-CDP antibodies as well as pre-immune serum were added to the reaction before the addition of radiolabeled probes. The arrows indicate SATB1 or CDP/Cux complexes. **C.** EMSA performed with BW5147, VL3 nuclear extracts, and radiolabeled E8III P2 probe. Anti-CDP antibodies and pre-immune serum were added to the reaction before the addition of radiolabeled probes. The arrow indicates CDP/Cux complexes.

3.2 Transgenic studies on the function of L2a element

In previous stable transfection studies, inclusion of the L2a element (as a 900 bp AccI/HpaI fragment) within their constructs significantly reduced the frequency of $CD8\alpha^+$ transfectants, suggesting that L2a may be a negative regulatory sequence on CD8 transcription [106]. Hostert and coworkers reported that a 4.3 kb HindIII/HindIII fragment containing DH cluster II, a fragment that is inactive in transgenic studies [52], allows the E8I enhancer to activate a reporter gene in DP thymocytes [56]. A transgenic approach was employed to further test if the L2a element, which is located within the first DH site of cluster II (CII-1), is responsible for modifying the specificity of the E8I enhancer *in vivo*.

3.2.1 Generation of transgenic mice expressing a CD8 reporter with or without L2a.

A hCD2 reporter gene was employed for the transgenic studies. It was derived from a mouse CD4 reporter gene construct produced by Sawada and coworkers [78] and 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 a human CD2 cDNA [212] with the SV40 polyadenylation site. Ellmeier *et al.* modified this reporter gene by replacing its CD4 promoter fragment with a polylinker, and a PCR-amplified mouse *CD8a* promoter [213] was inserted into the polylinker to make a construct called Tg-a [55].

Fig. 32A shows the schematic map of the constructs used for our L2a transgenic study. The production of transgenic constructs was carried out under collaboration with Dr. Wilfried Ellmeier, University of Vienna. Briefly, a PCR-amplified HindIII/ClaI *CD8a* promoter fragment was cloned into the pBluescript vector, and a 4.3 kb HindIII/HindIII DH cluster II fragment containing L2a

element was introduced upstream of the promoter. The 7.6 kb BamHI/BamHI fragment containing the E8I enhancer was subcloned upstream of 4.3 kb fragment, and a hCD2 reporter gene was inserted downstream of the promoter at the ClaI site. Transgene constructs were excised out of the vector by NotI and an 18 kb fragment was used for C57BL/6 pronuclear injections. Pronuclear injections were performed directly into C57BL/6 pronuclei. While much lower in efficiency, we employed C57BL/6 as recipients in order to eliminate strain effects and circumvent long backcross regiments. Southern hybridization was used to identify transgenic positive mice. The number of integrated copies was determined by comparing signal intensities of wild type and transgene-containing bands (Fig. 32B).

In addition to the wild type construct (L2aWT), a 4 kb DH cluster II fragment with deletion of the 210 bp L2a sequence was used to make the L2aD construct (Fig. 32A). A construct with a reversed orientation of the 4.3 kb L2a-containing fragment (L2aR) was also constructed.

3.2.2 CD8 reporter (hCD2) expression in both thymocytes and mature T cells is silenced in L2aWT transgenic mice

Fig. 32 shows the FACS analysis of thymocytes isolated from seven independent transgenic mice carrying the L2aWT transgene. Cells were stained with anti-CD4, anti-CD8a and anti-hCD2 antibodies, and then subjected to three-color FACS analysis. Expression of the hCD2 reporter gene on gated DP, CD4SP, and CD8SP subsets of thymocytes was plotted and analyzed in histograms.

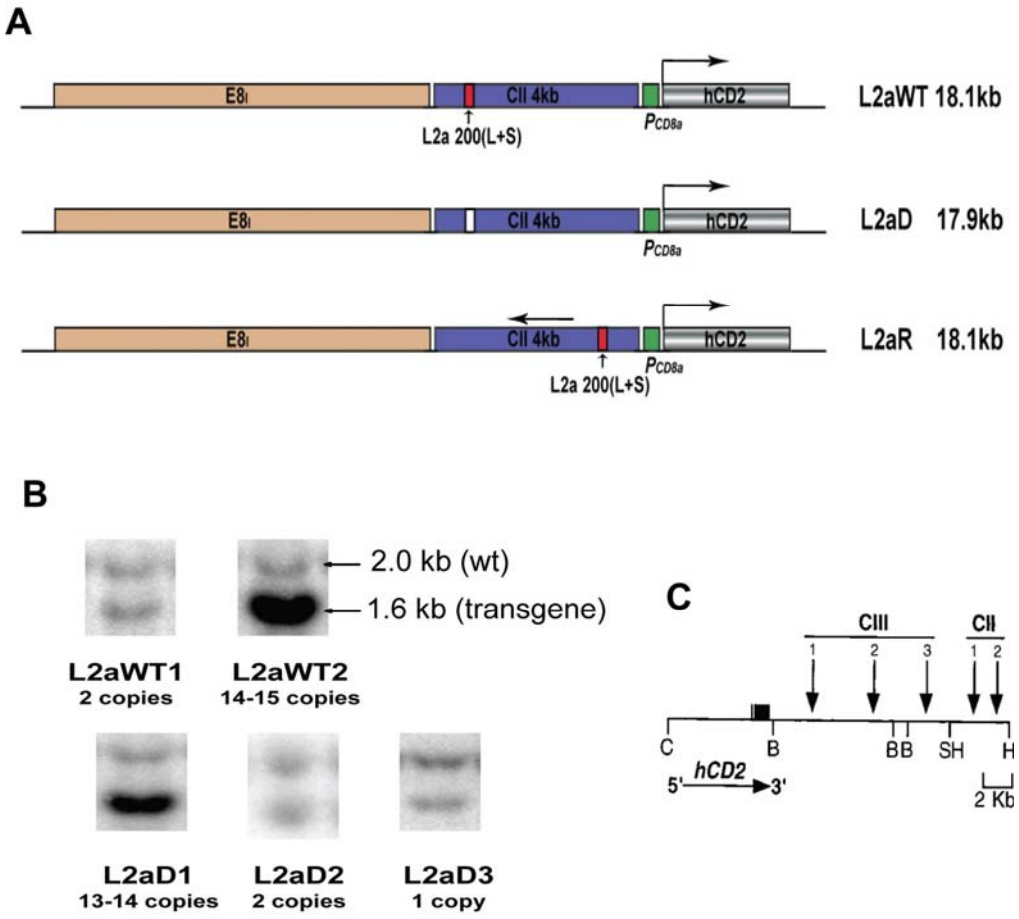


Fig. 32. Transgenic constructs designed to test the effect of L2a on E8I function.
A. Schematic of the transgenic constructs. Components of the constructs are highlighted in different colors and detailed in the text. The L2a element (red) resides in the first DH site of DH cluster II. L2aD has a deleted 200 (L+S) sequence in the HindIII/HindIII fragment and L2aR has an inverted one. **B.** Southern hybridization was used to identify positive transgenic mice, and the copy numbers were determined by comparing signal intensities of wild type and transgene bands. **C.** Transgenic construct used by Hostert and coworkers [56].

Surprisingly, six out of seven transgenic mice showed very low to undetectable levels of hCD2 expression in all three subsets of thymocytes. This contradicts results published previously [56]. These six transgenic strains lacked reporter expression in CD4SP or DP cells, but four of them showed significant hCD2 levels within a small portion (1-5%) of the CD8SP thymocytes. Because the E8I enhancer alone was shown to be sufficient for driving transgene expression in CD8SP cells [54, 55], this low frequency of cells capable of reporter expression level may suggest certain repressive effect on E8I from the L2a containing DH cluster II fragment.

The small group of hCD2 positive CD8SP cells observed in four L2aWT mice had reporter levels (MFI: 42-91) similar to that of L2aWT1 (MFI: 69). This suggests that the repressive effect of L2a may be silencing in the mode of a binary (on/off) switch. That is, rare cells that by-pass L2a-mediated silencing would have normally regulated CD8 transcription, resulting in variegated expression of CD8 reporter (as reported in our transgenes by hCD2) within the CD8SP subset. The L2a containing DH cluster II fragment may play a role as a silencer for the reporter expression driven by E8I enhancer.

Only one L2a-containing transgenic line, L2aWT1, expressed high levels of hCD2 in CD8SP (80.4%) and DP (80.3%) cells. This is the one exception from the L2aWT group of “escape” from the apparent repressive effects of L2a. We suspect that this results from a strong position (euchromatin) effect of L2aWT1 transgene integration.

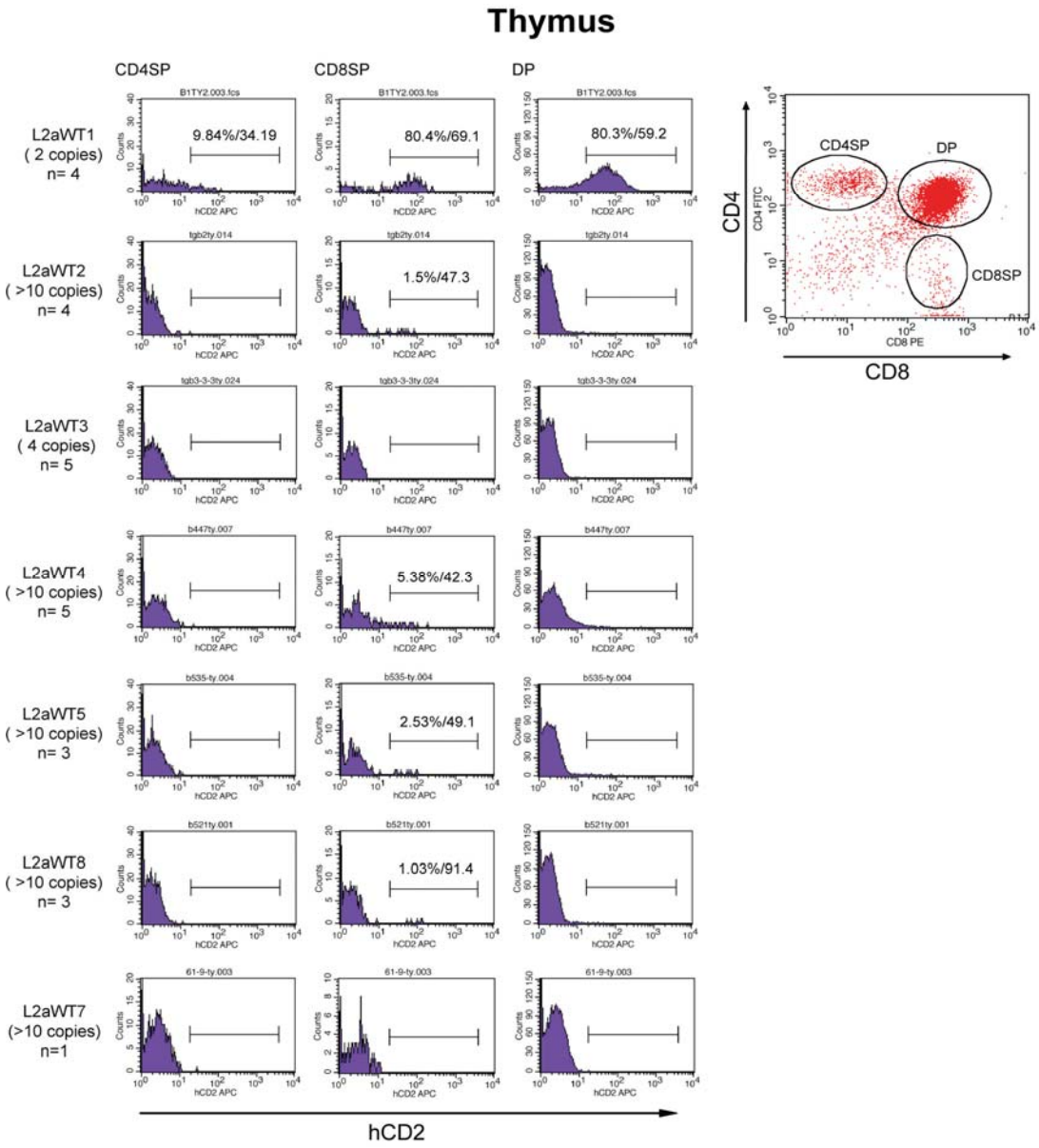


Fig. 33. Silenced reporter expression in thymocytes of L2aWT transgenic mice.
FACS analysis of thymocytes isolated from seven independent L2aWT transgenic mice. Cells were stained with anti-CD4, anti-CD8α and anti-hCD2 antibodies, and were applied to three-color FACS analysis. Expression of the hCD2 reporter gene on gated DP, CD4SP, and CD8SP thymocytes were analyzed in histograms. Percentages and MFIs of hCD2 positive (>1.0%) subsets and numbers (n) of mice tested are shown.

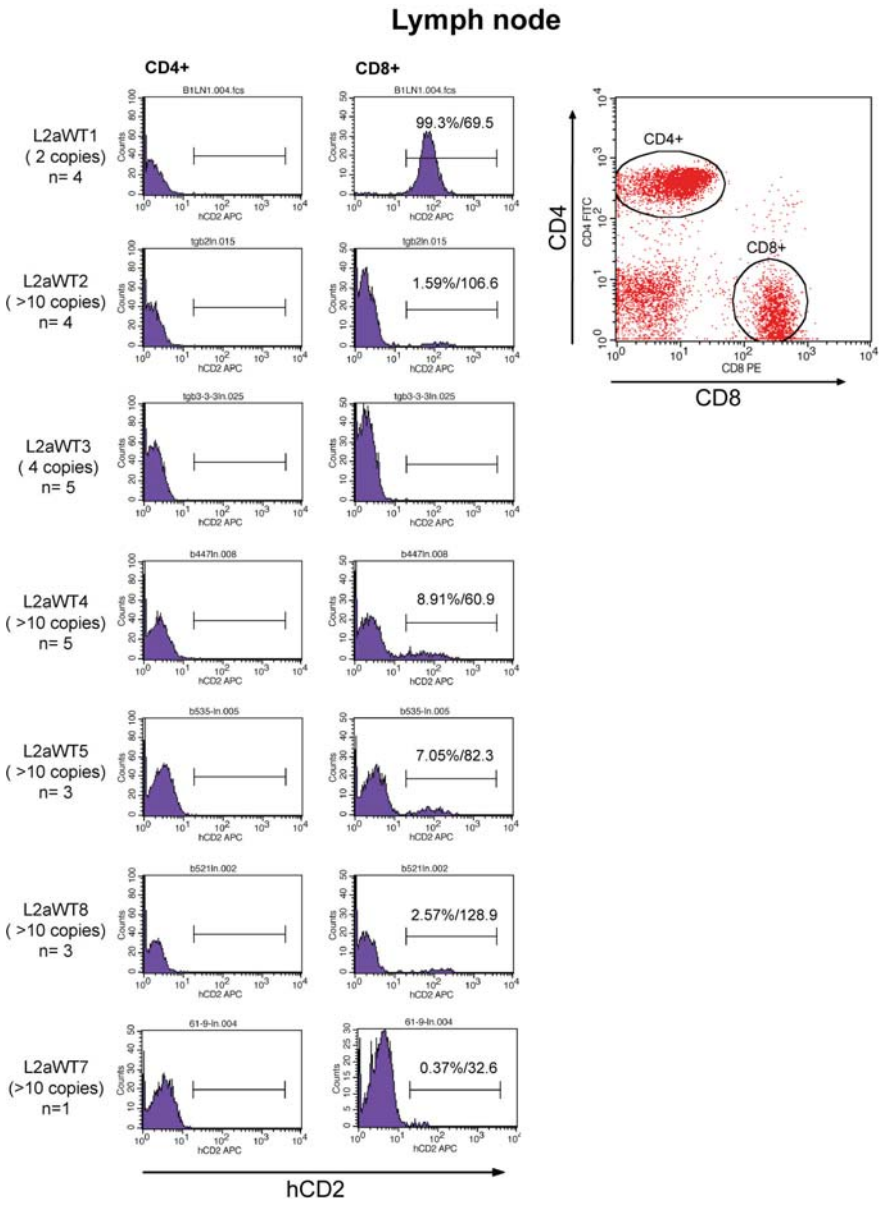


Fig. 34. Silenced reporter expression in lymph node T cells of L2aWT mice.
FACS analysis of lymph node T cells isolated from seven independent L2aWT transgenic mice. Cells were stained with anti-CD4, anti-CD8α and anti-hCD2 antibodies, and were applied to three-color FACS analysis. Expression of the hCD2 reporter gene on gated CD4 and CD8 lymphocytes were analyzed in histograms. Percentages and MFIs of hCD2 positive (>1.0%) subsets and numbers (n) of mice tested are shown.

Peripheral lymph node cells were also examined by three-color FACS analysis (Fig. 34). The CD8 positive cell subsets (except L2aWT1) showed similar hCD2 expression patterns as those observed in CD8SP thymocytes. Again, we observed a small population of hCD2 positive cells (2-9%) with significant MFI (61-107), consistent with the existence of a CD8 silencer that when circumvented provides variegated expression.

Neither CD4SP thymocytes (except L2aWT1) nor peripheral CD4 T cells expressed hCD2 on their surface, confirming the CD8 subset specificity of the transgenic E8I-driven construct. Among all L2a transgenic mice (including L2aWT mice and L2aD mice to be discussed below), each independent line expressed consistent levels of hCD2, due (we suspect) to the pure C67BL/6 background. No significant variation of reporter expression was observed in any transgenic line after 2-3 generations.

3.2.3 CD8 reporter (hCD2) expression in both thymocytes and mature T cells is rescued by deletion of L2a in L2aD transgenic mice.

Fig. 35 shows the FACS analysis of hCD2 expression on isolated thymocytes from the L2aD transgenic mice in which the L2a element has been deleted. Significant expression was observed in CD8SP and DP thymocytes in four out of five L2aD mice. This is consistent with observations by Hostert and coworker [56] showing that the DH cluster II fragment (lacking L2a) facilitates E8I enhancer-driven reporter expression in DP thymocytes. Combined with the results from L2aWT transgenic mice (Fig. 33), the data indicate that the L2a element is the DNA sequence that mediates all the silencing observed.

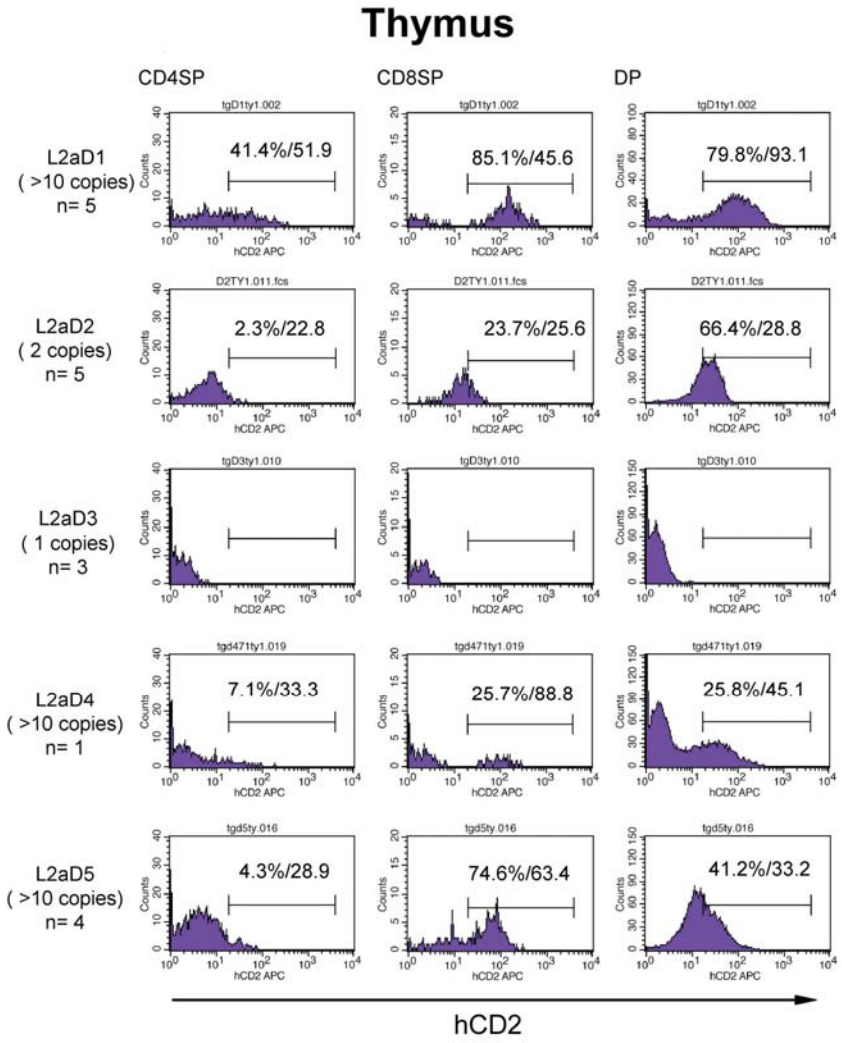


Fig. 35. DH cluster II without L2a restores reporter expression in DP thymocytes of L2aD transgenic mice.

FACS analysis of thymocytes isolated from five independent L2aD transgenic mice. Cells were stained with anti-CD4, anti-CD8α and anti-hCD2 antibodies, and were applied to three-color FACS analysis. Expression of the hCD2 reporter gene on gated DP, CD4SP, and CD8SP thymocytes were analyzed in histograms. Percentages and MFIs of hCD2 positive (>1.0%) subsets and numbers (n) of mice tested are shown.

Another potentially informative observation was that CD4SP thymocytes from these four L2aD transgenic mice expressed detectable levels of hCD2 on their surface (Fig. 35). This suggests that this apparent of loss of E8I-mediated subset specificity is lost if L2a silencing is lost. L2aD3 was the only strain that did not express a transgene on any thymocyte subset in this group. This may be the result of transgene integration into a constitutively silenced heterochromatic region. Analysis of mature T cells from lymph nodes showed similar robust CD8 reporter expression in the CD8 positive cells (Fig. 36).

Since the L2a element was not required for driving hCD2 expression in DP thymocytes, other *cis*-acting DNA elements may be responsible for this function. Two additional, strongly binding SATB1 sites have been identified (Results section 3.1.7), and these may be good candidates for further study.

Why our results differ from those obtained by Hostert and coworkers [56] may be revealed by a closer comparison of the transgenes employed (Fig. 32C). Hostert *et al*. placed the E8I enhancer (CIII) and DH cluster II downstream of the hCD2 reporter. This is the opposite (3' with regard to CD8 transcription) side of the germline configuration and the configuration of our construct. Although some reports [54-57] suggest that the orientation of CD8 enhancer(s) is not a factor affecting reporter expression, this may not be the case for L2a. In the single line (L2aR) that we created in which the orientation of L2a was reversed, hCD2 was strongly expressed in both CD8SP and DP thymocytes (data not shown). This suggests that the silencing function of the L2a element is dependent on either position, orientation, or both.

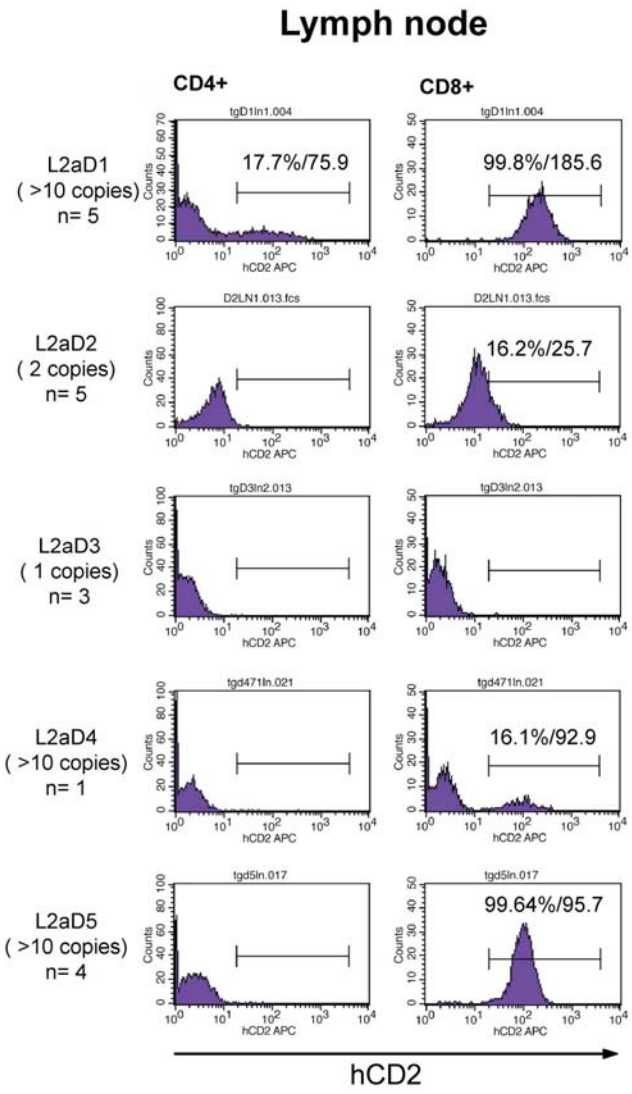


Fig. 36. DH cluster II without L2a allows reporter expression in CD8 positive T cell in L2aD transgenic mice.

FACS analysis of lymph node T cells isolated from five independent L2aD transgenic mice. Cells were stained with anti-CD4, anti-CD8α and anti-hCD2 antibodies, and were applied to three-color FACS analysis. Expression of the hCD2 reporter gene on gated CD4 and CD8 lymphocytes were analyzed in histograms. Percentages and MFIs of hCD2 positive (>1.0%) subsets and numbers (n) of mice tested are shown.

3.2.4 Variegated reporter gene expression in L2aWT transgenic mice is stable.

Splenocytes from L2aWT5 and L2aD5 transgenic mice were labeled with anti-CD8α MicroBeads (MACS System), and $CD8^+$ cells were positively separated as described in Materials and Methods. Purified CD8 cells were treated with pronase (Sigma) and placed in culture at 37^oC or 4^oC as controls for 18 hours. As shown in Fig. 37, hCD2 expression on both L2aWT5 and L2aD5 sorted cells was up-regulated efficiently 18 hours after the pronase treatment. This result indicates that the transgenes have retained their ability to re-express the reporter molecules. Importantly, it allows us to conclude that the small fraction of $hCD2^+$ cells in the presence of L2a (in L2aWT5) is the product of variegated expression. Some of the hCD2 molecules expressed on the cell surface were retained after treatment, suggesting they were more resistant to pronase than were CD8 molecules.

3.2.5 Expression of CD8 reporter transgenes by $CD8\alpha\alpha^+$ IELs is similar to that of conventional $CD8\alpha\beta^+$ T cells

It is known that the CD8αα homodimer is expressed in IELs within the small intestine and in a subset of dendritic cells (DCs) [90]. To determine if the hCD2 gene, which is driven by the E8ı enhancer and *CD8a* promoter, is co-expressed on the cells bearing the CD8αα homodimer, IELs were isolated from both L2aWT and L2aD transgenic mice and were tested for reporter expression. IELs were stained with either the combination of anti-CD8α, anti-CD8β and anti-hCD2 antibodies or the combination of anti-TCRγδ, anti-CD8β and anti-hCD2 antibodies. Gated IEL subsets were analyzed for reporter expression (Fig. 38).

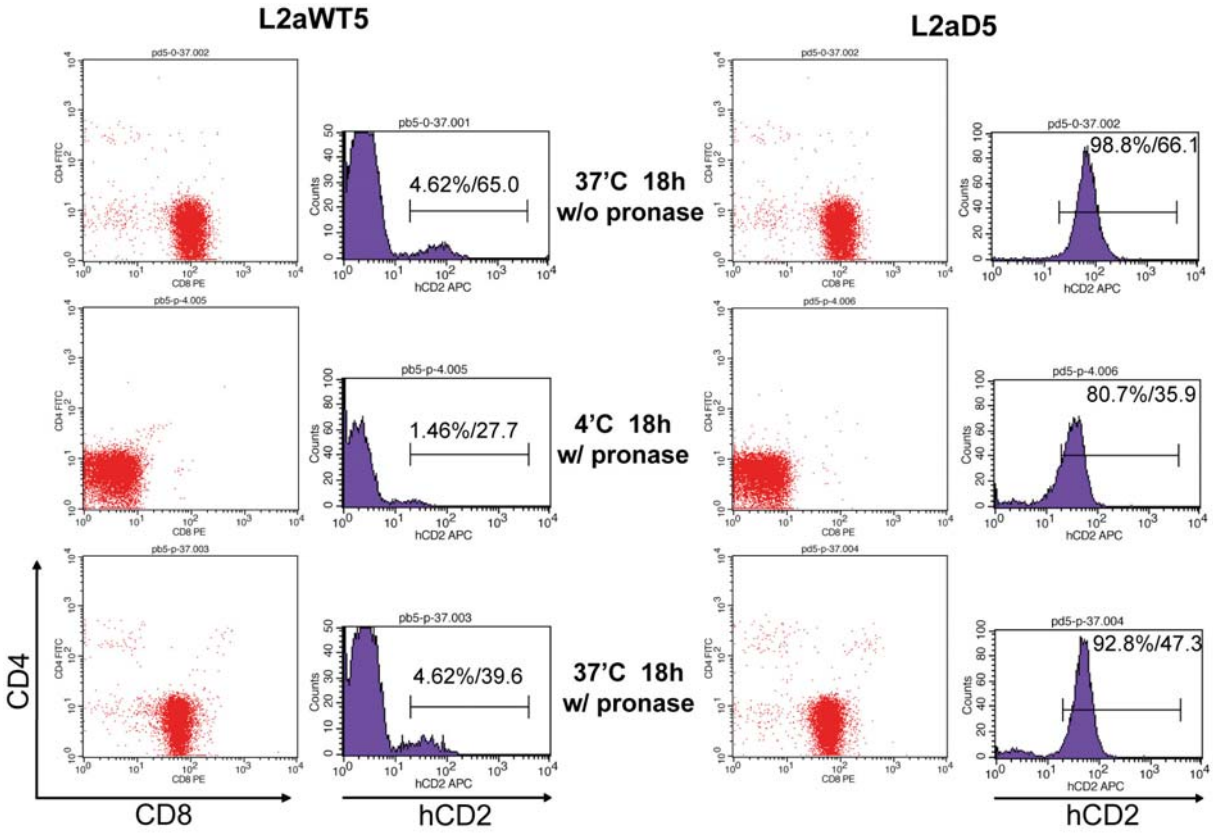


Fig. 37. Variegated expression of reporter gene in L2aWT mice is stable.
The sorted $CD8^+$ populations were treated with pronase and placed in culture at 37°C for 18 hours or 4°C as controls. hCD2 and CD8 expression on both L2aWT5 and L2aD5 sorted cells were upregulated efficiently 18 hour after the pronase treatment. Percentages and MFIs of hCD2 positive subsets are shown.

As shown in Fig. 38, the hCD2 levels within both CD8αα$^+$ and CD8αβ$^+$ IELs isolated from the L2aWT3, 4 and 5 strains showed similar silencing as those isolated from lymph node T cells. Furthermore, variegated reporter expression was also observed in IELs of L2aWT mice. Fig. 39 shows the hCD2 expression pattern observed in IELs isolated form L2aD mice which lack the L2a element in the transgene. Expression was observed on both CD8αα and CD8αβ subsets at levels comparable levels to that on lymph node CD8$^+$ cells. This result is consistent with previously published results that E8I enhancer directs the reporter expression on IELs [57]. Observations from both groups of mice support the conclusion that the L2a element is a silencer that controls CD8 expression (both CD8αα and CD8αβ) in IELs as well as in thymocytes and mature T cells.

Comparing all the cell subsets tested for L2aWT and L2aD transgenic mice, the expression pattern (both percentage of positive cells and MFI) of the hCD2 reporter in IELs is similar to those of thymocytes and CD8$^+$ T cells. This implies that CD8 expression in IELs may be regulated under a similar mechanism as in thymocytes. Our evidence weighs in favor of a common thymic lineage origin and against the long-time debate [214] regarding extrathymic origin of the IELs.

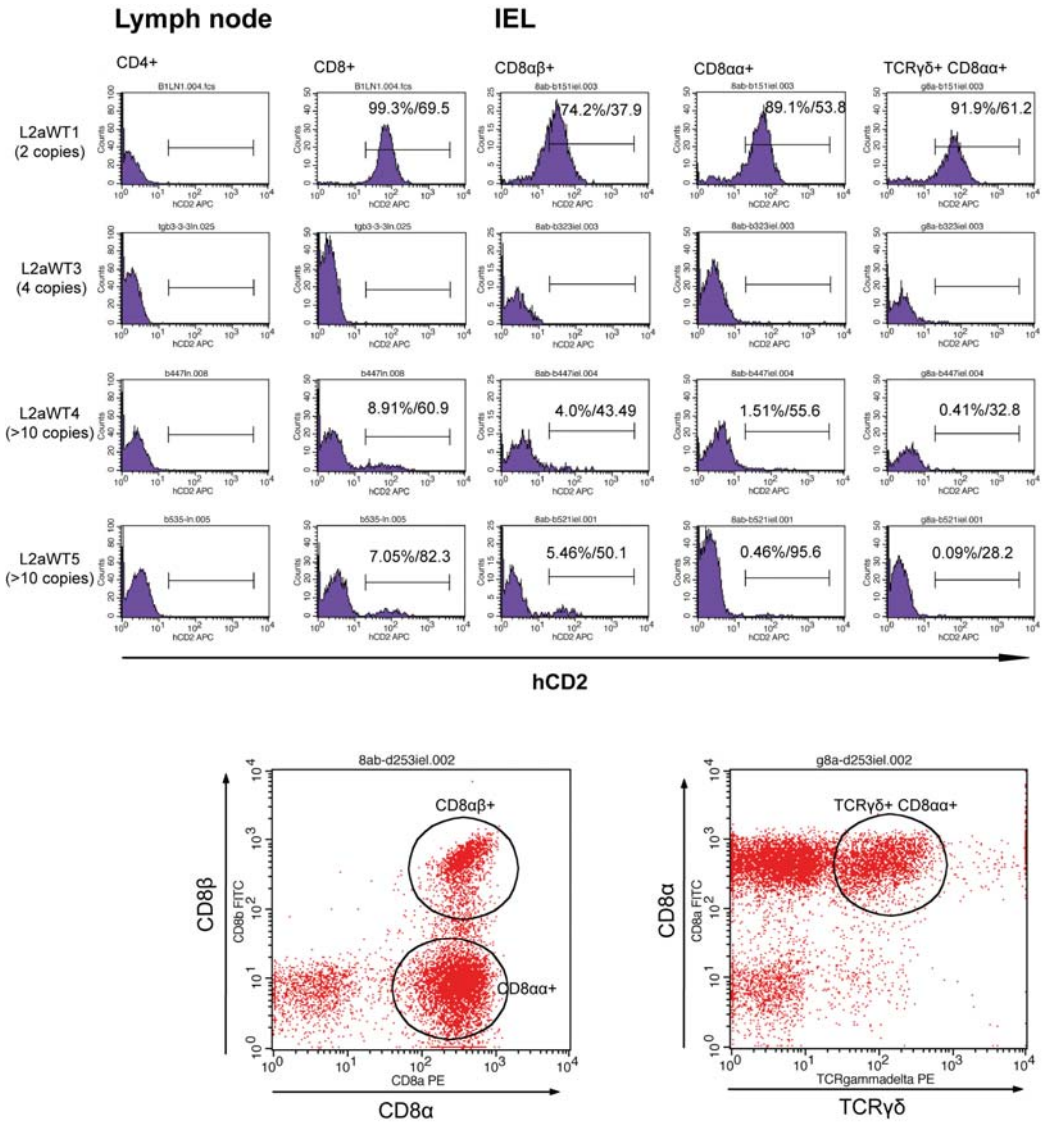


Fig. 38. Expression of hCD2 reporter gene on IELs is similar to those of CD8 positive T cells in L2aWT mice.

IELs were isolated from four L2aWT transgenic mice, and were stained with either the combination of anti-CD8α, anti-CD8β and anti-hCD2 antibodies (left)or the combination of anti-TCRγδ, anti-CD8β and anti-hCD2 antibodies (right). Gated IEL subsets were analyzed for hCD2 reporter expression. Percentages and MFIs of hCD2 positive (>1.0%) subsets are shown.

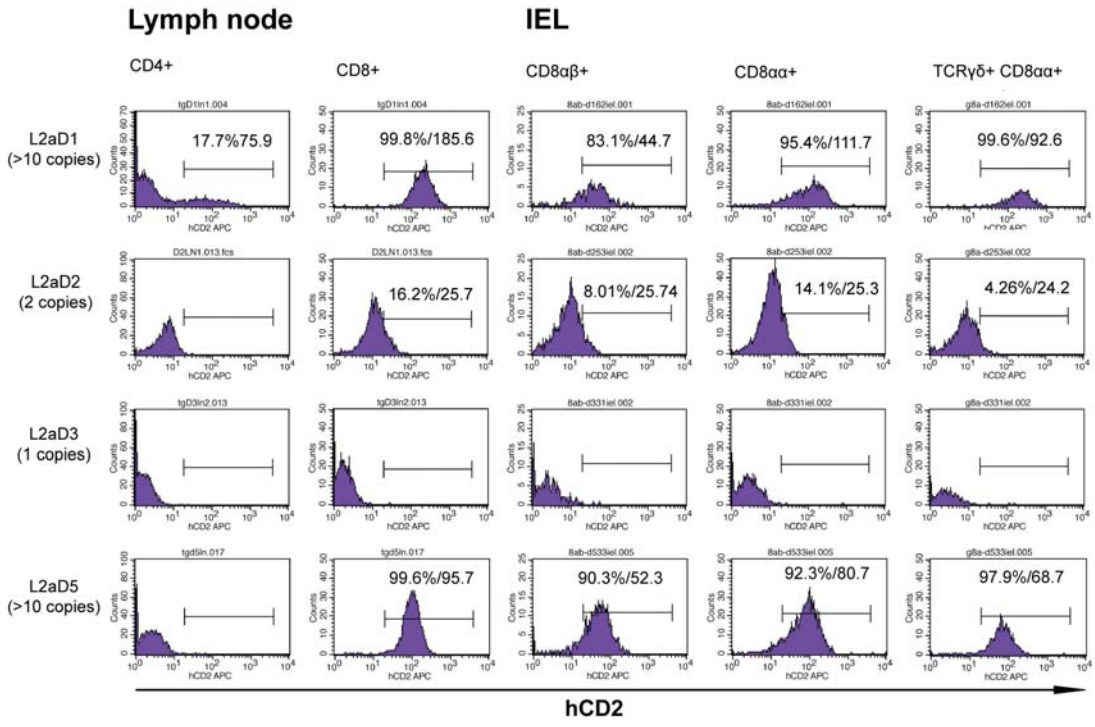


Fig. 39. Expression of hCD2 reporter gene on IELs is similar to those of CD8 positive T cells in L2aD mice.

IELs were isolated from four L2aD transgenic mice, and were stained with either the combination of anti-CD8α, anti-CD8β and anti-hCD2 antibodies or the combination of anti-TCRγδ, anti-CD8β and anti-hCD2 antibodies. Gated IEL subsets were analyzed for hCD2 reporter expression. Percentages and MFIs of hCD2 positive (>1.0%) subsets are shown.

3.2.6 CD8 transgene reporter expression differs between dendritic (DC) cells and CD8 positive T cells

In the mouse spleen, a subset of DCs expresses CD8αα homodimers. These CD8αα$^+$ DCs were originally considered lymphoid progenitors [215], but more recent results indicate that they have developed from both myeloid and lymphoid progenitors [90]. To test whether the E8$_I$ enhancer and DH cluster II fragment-containing L2a can regulate hCD2 expression in the DCs, splenocytes were isolated from L2aWT8 and L2aD5 mice and were analyzed by FACS. Cells were stained with anti-CD11c, anti-CD8α and anti-hCD2 antibodies, and the analysis of gated cells is shown in Fig. 40.

The CD11c$^+$CD8α$^+$ DCs from L2aWT8 and L2aD5 expressed detectable levels of the hCD2 reporter, but expression was much lower than that in CD11c$^-$CD8$^+$ T cells. The MFIs of CD8α on CD11c$^+$CD8α$^+$ DCs and CD11c$^-$ CD8α$^+$ splenocytes were similar (data not shown). Thus, the regulation of CD8αα expression in DCs may differ from that of T cells, or additional *cis*-acting elements may be needed for this regulatory process.

3.2.7 CD8 transgenic reporter expression is modulated via TCR signaling

Recent studies showed that CD8αα molecules are transiently induced in a selected subset of CD8αβ$^+$ cells upon antigenic stimulation, and they promote the survival and differentiation of activated lymphocytes into memory CD8 T cells [102]. E8$_I$ enhancer knockout mice do not produce these cells, suggesting that the induction of CD8αα on activated CD8αβ$^+$ splenocytes is controlled by the E8$_I$ enhancer [102]. Since the hCD2 reporter of our transgenic constructs is under the control of the E8$_I$ enhancer and the L2a DH cluster II fragment, we felt it would be informative to test the response of the reporter gene to TCR antigenic stimulation.

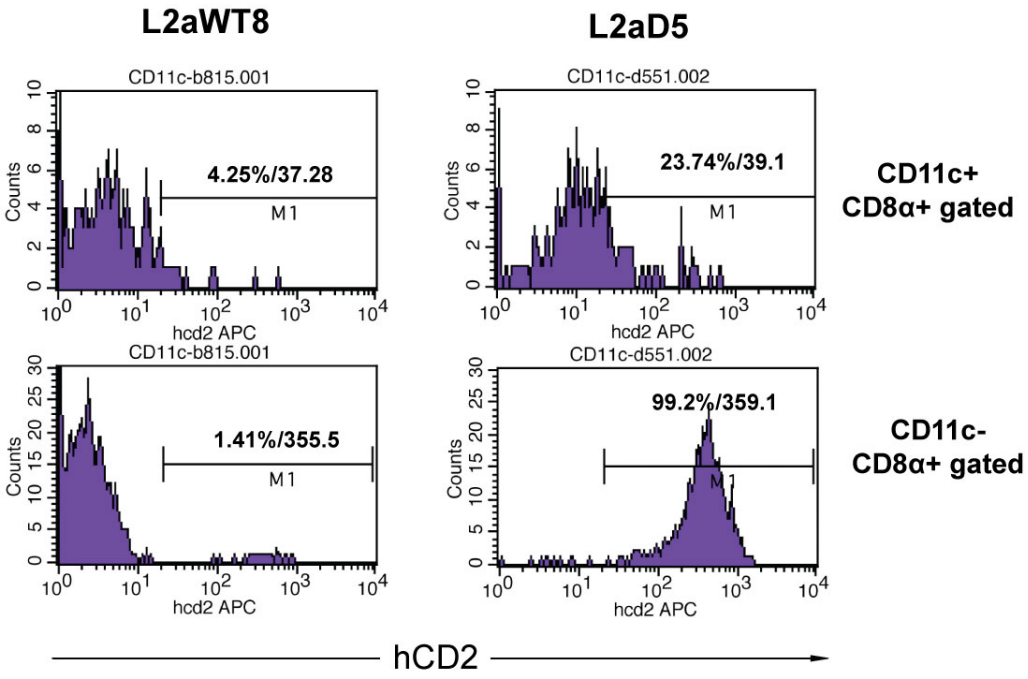


Fig. 40. CD8 reporter expression on dendritic cells differs from that on CD8 positive T cells in L2a transgenic mice

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.

Splenocytes from both L2aWT and L2aD transgenic mice were cultured with or without purified anti-CD3ε antibody, and expression of the hCD2 reporter was analyzed by FACS on day 4 (Fig. 41). All three L2aWT mice tested showed decreased reporter expression (reduced MFI) upon anti-CD3 stimulation, but the hCD2 levels were not affected in splenocytes from L2aD mice. It appears that the TCR activation strengthened the silencing of hCD2 expression by L2a, and deletion of L2a abolishes further silencing caused by simulation. These results suggest that the L2a element may function as a silencer in CD8αα-regulated memory T cell selection.

3.2.8 Reduced levels of SATB1 or CDP/Cux proteins cause modest changes in CD8 transgene expression.

To examine the effects of SATB1 and CDP/Cux on the regulation of CD8 expression in the presence and absence of L2a, we bred L2aWT and L2aD transgenic mice with previously established SATB1-reduced [158] and CDP/Cux knockout (ΔC) [201] mice (kindly provided by Dr. Jaquelin Dudley).

To cross the L2a transgene onto the SATB1-reduced background, L2aWT and L2aD mice were bred to homozygous SATB1 antisense (As/As) transgenic mice to obtain L2a/+, As/+ mice. One backcross with As/As mice produced L2a/+, As/As mice. Then these mice were bred to SATB1-reduced mice (As/As, SATB1 KO/+), such that 25% of the offspring were the desired L2a/+, As/As, SATB1 KO/+ genotype.

The homozygote of ΔC mice is lethal, but the heterzygous mice show significantly reduced (less than 50%) CDP/Cux expression in the thymus and mammary glands [201]. To generate the CDP/Cux reduced L2a transgenic mice, L2aWT and L2aD mice were crossed to heterozygous ΔC mice. The desired genotype is L2a/+, CDP KO/+.

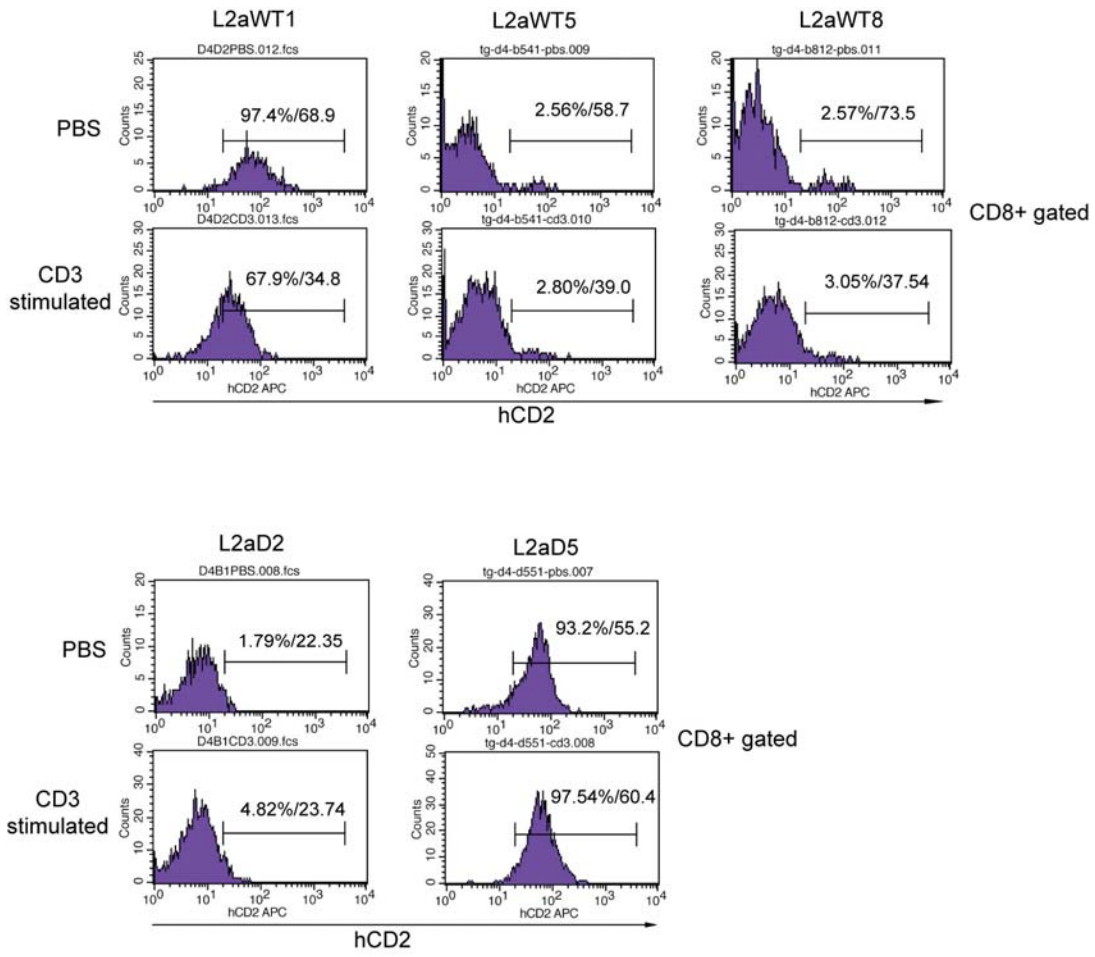


Fig. 41. Expression of CD8 transgenic (hCD2) reporter is modulated by TCR signaling.
Splenocytes from three L2aWT and two L2aD transgenic mice were cultured with or without purified anti-CD3ε antibody, and expression of the hCD2 reporter were analyzed by FACS on day 4. Percentages and MFIs of hCD2 positive (>1.0%) subsets are shown.

When the L2aWT2 mice were crossed onto a SATB1 knockdown background, the small fraction of both CD8SP thymocytes and CD8 T cells demonstrating variegated hCD2 expression was decreased (Fig. 42). This indicates that SATB1 is involved in re-starting silenced CD8 expression mediated by L2a. As shown in both Figs. 42 and 43, expression of the hCD2 reporter (MFI) decreased modestly on all CD8-expressing subsets, especially in DP thymocytes under the reduced SATB1 background. Both L2aWT and L2aD transgenic mice showed decreased hCD2 levels, indicating that SATB1 is required for maintaining reporter expression. Since other *cis*-acing elements in the DH cluster II are responsible for driving CD8 reporter expression in DP thymocytes, these unknown elements may the targets of SATB1.

Thus, SATB1 may play two roles in regulating CD8 expression. It functions to overcome L2a silencing at certain developmental stages to assist in re-expression of CD8, such as the transition from $CD4^{+}CD8^{low}$ to CD8SP cells during the corecepter reversal process. Meanwhile, once CD8 expression is established, SATB1 may work with other *cis*-acting elements within DH cluster II to maintain the expression of CD8.

The expression of hCD2 in CDP/Cux reduced mice also showed modest changes (Fig. 44). Increased reporter levels were observed in CD8 thymocytes and mature T cells, suggesting a possible negative regulatory function of CDP/Cux.

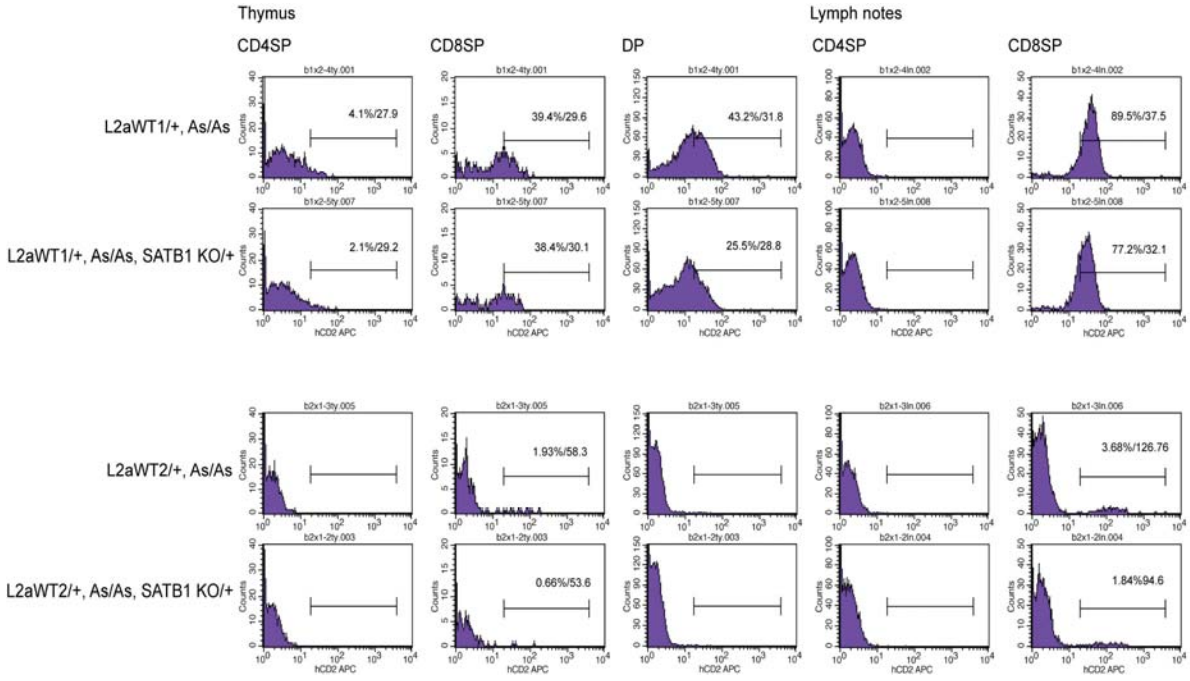


Fig. 42. Reduced levels of SATB1 protein results in modest changes in CD8 reporter expression in L2aWT mice

L2aWT1 and L2aWT2 transgenic mice were crossed to SATB1 reduced mice. Expression of the hCD2 reporter on thymocytes and lymph node T cells was analyzed by FACS. Percentages and MFIs of hCD2 positive (>1.0%) subsets are shown

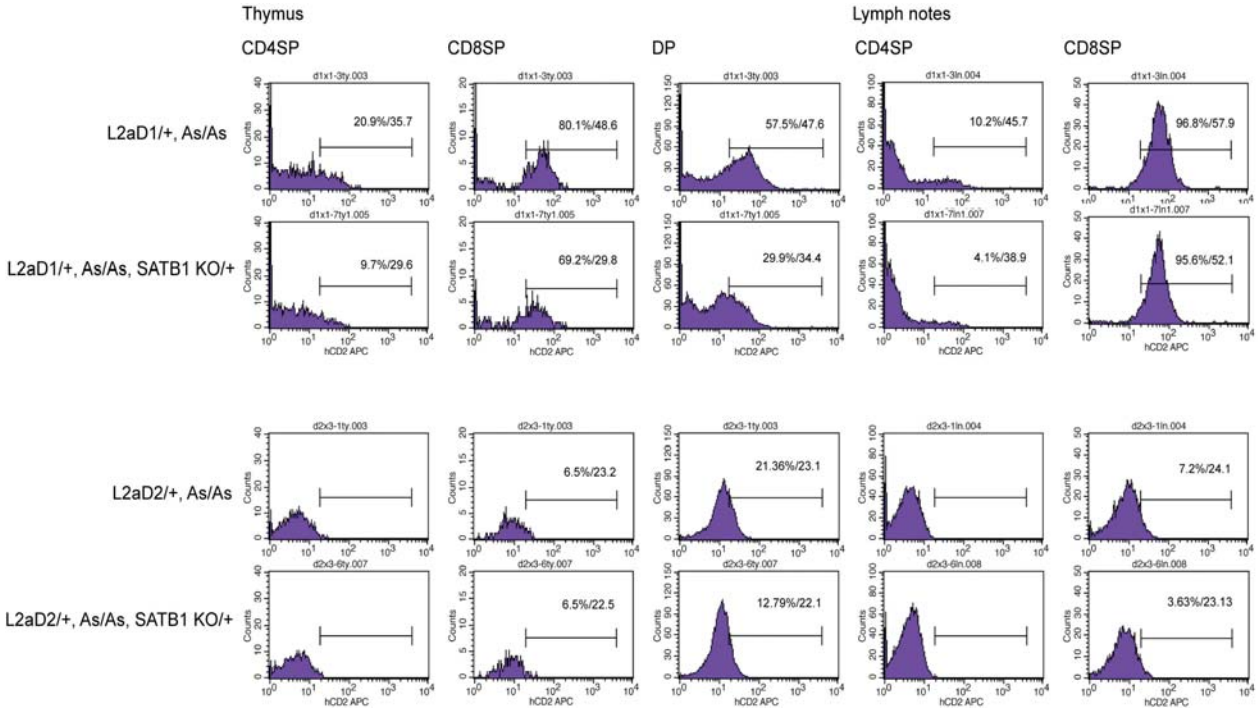


Fig. 43. Reduced levels of SATB1 protein causes modest changes in CD8 reporter expression in L2aD mice

L2aD1 and L2aD2 transgenic mice were crossed onto SATB1-reduced mice to place the L2a transgene onto the reduced SATB1 background. Expression of the hCD2 reporter on thymocytes and lymph node T cells were analyzed by FACS. Percentages and MFIs of hCD2 positive (>1.0%) subsets are shown.

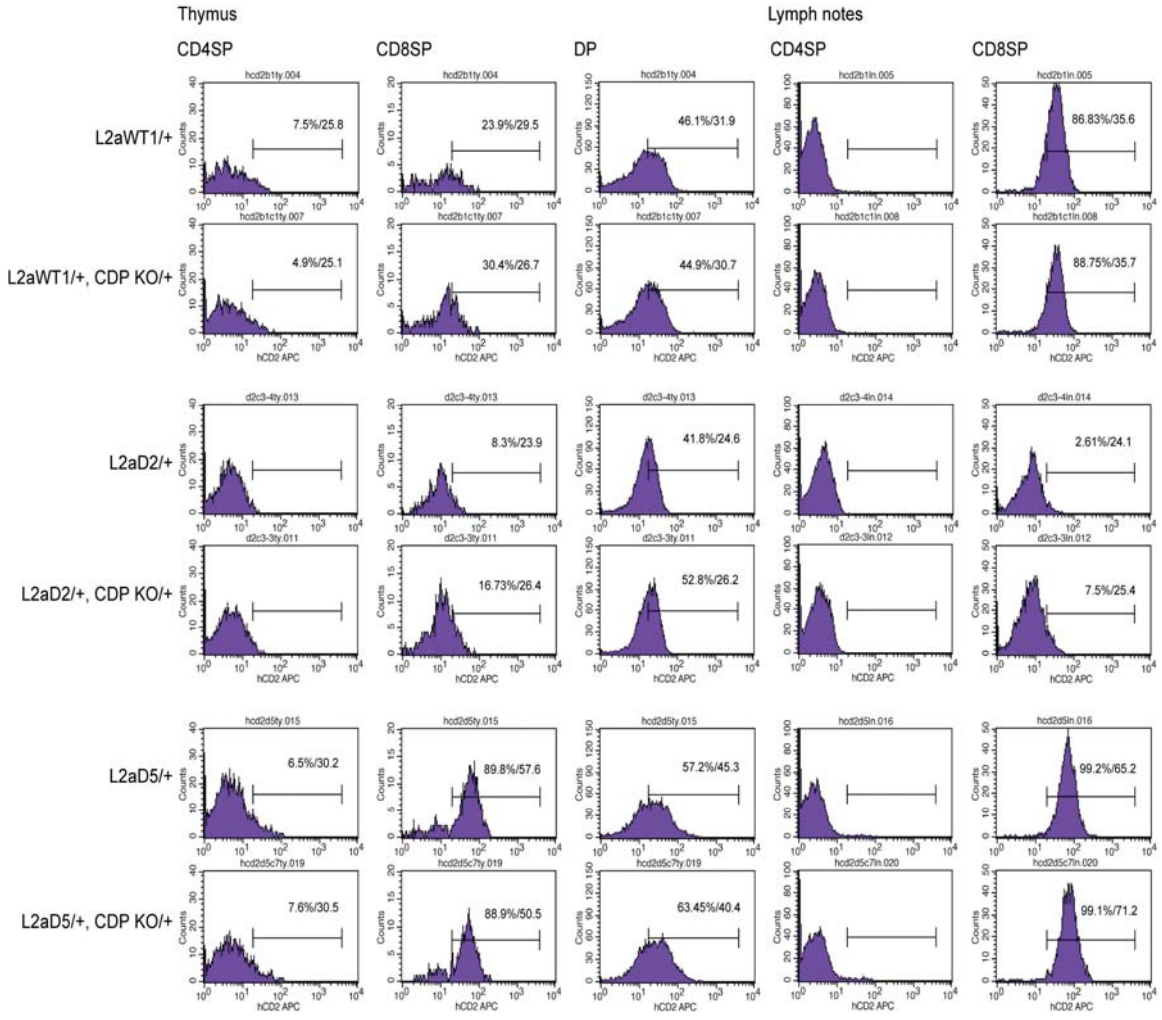


Fig. 44. Reduced levels of CDP/Cux protein cause modest changes in CD8 reporter expression in L2aWT and L2aD mice.

L2aWT and L2aD transgenic mice were crossed onto CDP/Cux knockout (ΔC) mice to place the L2a transgene onto the reduced CDP/Cux background. Expression of the hCD2 reporter on thymocytes and lymph node T cells was analyzed by FACS. Percentages and MFIs of hCD2 positive (>1.0%) subsets are shown.

3.3 Targeted deletion/knock-in studies on the function of the L2a element

It has been reported that a targeted deletion of a 3.4kb genomic region (DH cluster CII-1 and CII-2) containing the L2a element led to abnormal thymocyte development characterized by a large population of thymocytes that never turn on *CD8* gene expression and a decrease in the thymic and peripheral CD8SP T cell population [1]. Our transgenic studies reported above suggest that the L2a element may be a silencer of *CD8* gene transcription. To further investigate the putative role of L2a in the natural chromosomal context *in vivo*, knock-in mice were produced with deletion or mutations in the L2a element.

3.3.1 Generation of the L2a Knock-in mice

The targeting construct was generated as shown in Fig. 45. Two genomic fragments were cloned into a pOSDupDel vector (Open Biosystems) containing the HSV-tk gene (not shown). The left homology arm of the construct is a 2.3kb SphI-BamHI fragment of the wild type locus. The longer right homology arm is a 7.8kb BamHI-NotI fragment. Between the two arms, there is a neomycin resistance gene driven by the thymidine kinase promoter flanked by two *loxP* sites. The L2a wild-type or L2a mutated region is located on the right arm of the targeting construct.

The knock-in constructs were linearized by NotI and transfected into 129SvEV embryonic stem cells using electroporation. Southern hybridization was used to screen the recombinant clones using probe 1 or 2 for left arm or right arm, respectively (Fig. 45A, B). High recombination efficiencies in ES cells (KI-WT 8.6%; KI-ΔL2a 23.8%; KI-M1 21.4%) were observed, indicating that L2a might be located in euchromatic, accessible chromatin associated with active transcription of genes.

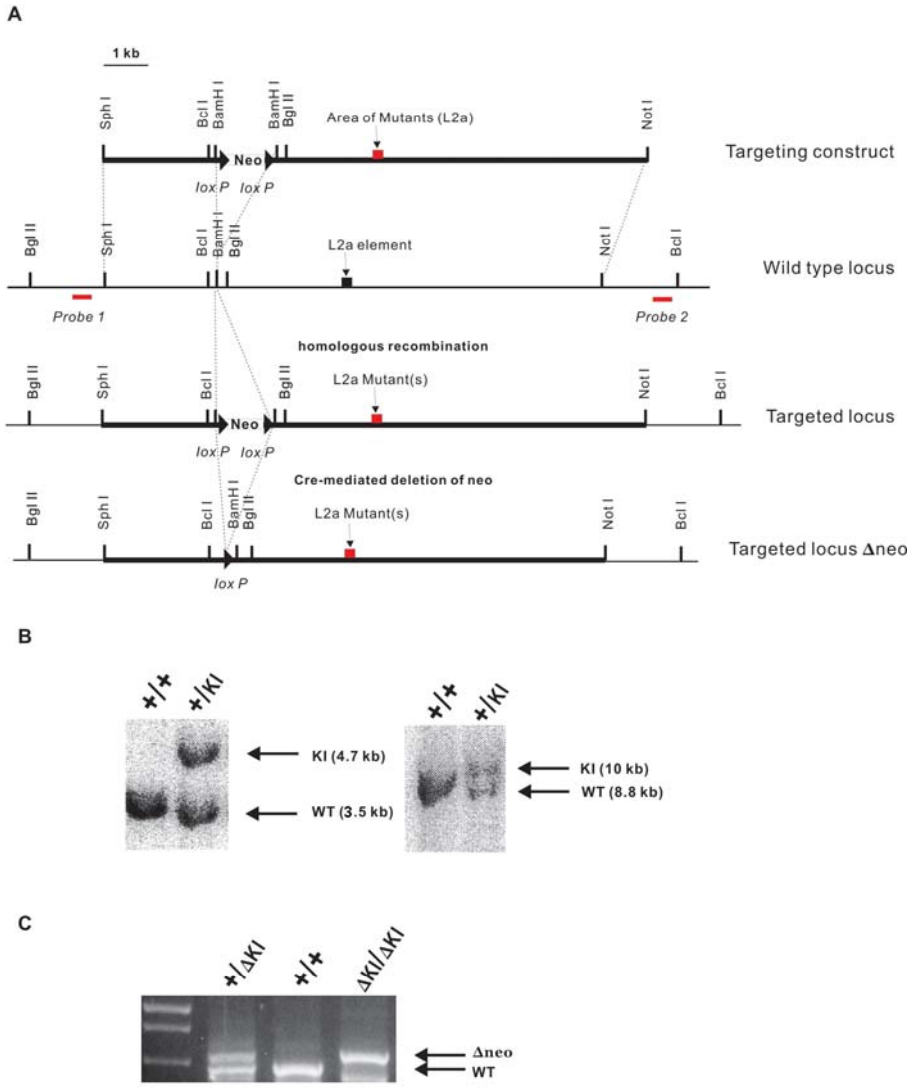


Fig. 45. L2a knock-in strategy and analysis.
A. Schematic of the targeting construct, the CD8 locus before and after homologous recombination, the targeted locus, and the genomic locus after Cre recombinase-mediated deletion of the *neo* gene. **B**. Southern blots of *Bgl* II (left) and *Bcl* I (right) digested DNA isolated from a wild-type 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 *lox*P sites are flanked by primers, and there is a 40 bp difference between wt and Δneo bands.

Selected positive ES clones were injected into day 3.5 C57BL/6J blastocysts and transferred into (B6/D2) F1 pseudopregnant females. Male chimeric mice were backcrossed to C57BL/6 females, and agouti progeny were screened for germline transmission of the targeted gene by Southern blot analysis of tail DNA (Fig. 45B). Since the presence of the *neo* cassette could influence the results [2], mice carrying the targeted allele were crossed with mice that expressed Cre under ubiquitous E2A transcriptional control to delete the *neo* gene. After the removal of the neo cassette, PCR was used to confirm the neo-deleted allele (Fig. 45C).

To investigate the function of the L2a element, more subtle modifications were introduced within specific sequences associated with binding proteins. Wild type L2a knock-in mice (KI-WT) were created as a control and the 200 (L+S) deletion of L2a was introduced to make the KI-ΔL2a knock-in mice. Two L2a mutants, M1 and M4, generated in previous study [3] (Fig. 46) were subcloned into the knock-in targeting construct to make KI-M1 and KI-M4 mice. The M1 mutant contains mutations that abolish the binding of SATB1, but leave the binding of CDP/Cux intact; The M4 mutant binds SATB1 strongly and interacts with CDP/Cux very weakly. KI-WT, KI-M1 and KI-ΔL2a mice have been created for this study, and KI-M4 mice are under construction.

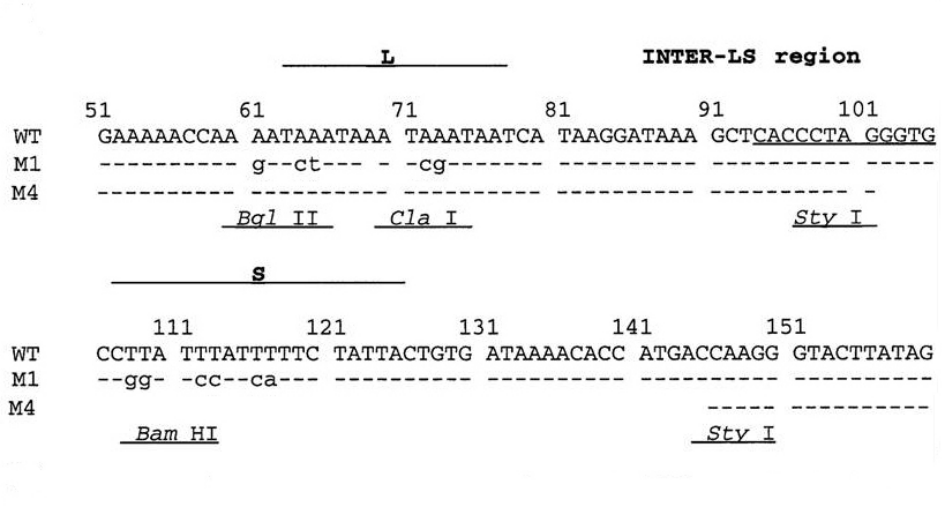


Fig. 46 L2a mutants for loss of SATB1 (M1) and CDP/Cux (M4) employed in knock-in studies.

Previously created mutants M1 and M4 were cloned into the knock-in targeting construct to make KI-M1 and KI-M4 mice. M1 abolishes the binding of SATB1, but leaves the binding of CDP/Cux intact; M4 binds SATB1 strongly and interacts with CDP/Cux very weakly.

3.3.2 Expression of CD8 is not affected in thymocytes and peripheral T cell subsets in mice lacking the SATB1 binding site (KI-M1) within L2a.

Lymph node cells and thymocytes were isolated from homozygous KI-WT and KI-M1 mice, stained with antibodies against CD4 and CD8α, and then analyzed by FACS. As shown in Fig. 47, the introduction of the M1 mutation (loss of SATB1 binding to L2a) did not alter the CD8 expression in thymocytes or lymph node T cells from KI-M1 mice. At least five pairs of mice were tested and none of them showed any significant changes.

Different 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 (Fig. 48). RT-PCR was also used to measure CD8α transcription levels in both mice, and no significant changes were observed (data not shown). These results indicate the M1 mutation that abolishes SATB1 binding to L2a does not affect CD8 expression and T cell development.

In addition, comparisons between KI-WT mice and C57BL/6x129/Sv mice indicated no differences in CD4, CD8 expression as well as other surface makers. This means that the inclusion of recombined *loxP* sites in KI-WT mice has no effect on phenotype, and can be used as a control for KI-mutant mice.

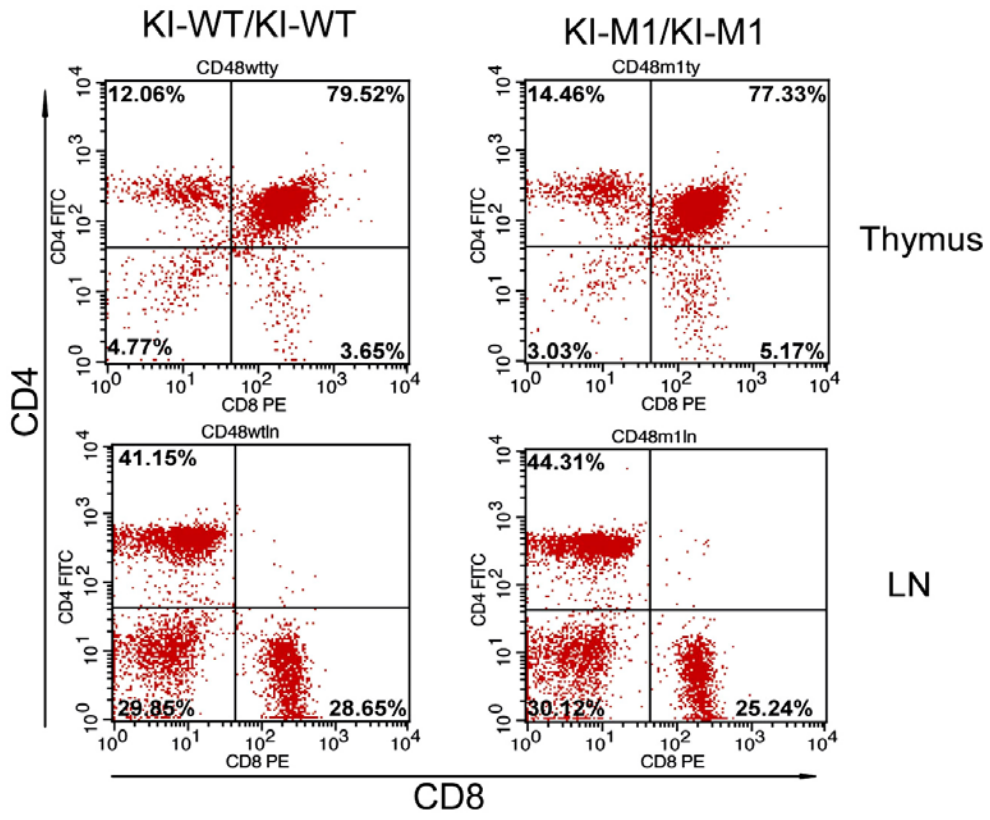


Fig. 47. CD8 expression is unaffected in thymocytes and lymph node T cells from KI-M1 mice.

Lymph node cells and thymocytes were isolated from homozygous KI-WT and KI-M1 mice and stained with antibodies against CD4 and CD8α molecules, and the cells were analyzed by FACS. Percentages of each cell population were shown.

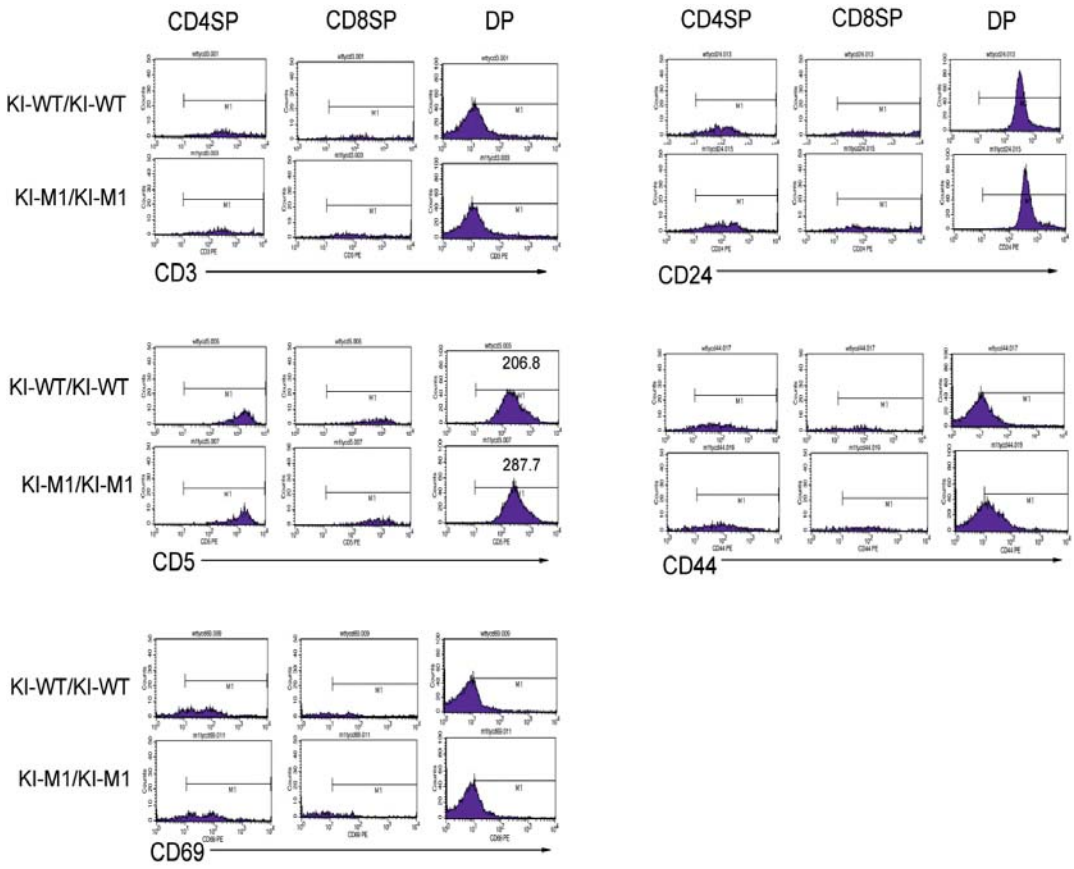


Fig. 48. Expression of T cell surface markers is unaffected in KI-M1 mice.
The indicated developmental T cell surface markers were tested on CD4SP, CD8SP and DP thymocytes. KI-WT and KI-M1 mice showed similar expression levels of CD3, CD5, CD24, CD44 and CD69 molecules.

3.3.3 T cells carrying complete L2a deletion (KI-ΔL2a) show modest changes in CD8 and CD4 expression

Three out of five KI-ΔL2a homozygous mice showed changes in both CD4 and CD8 expression in thymocytes and lymph node T cells, whereas the other two showed no difference. Fig.49 shows the results from one of the three homozygous KI-ΔL2a mice. Both CD4SP and CD8SP thymocyte subsets were increased by about 50% (9.9% to 16.9%, 2.5% to 4.0%). CD4 and CD8 T cells in lymph nodes both increased modestly (15-20%). The MFIs of CD4 and CD8 were the similar in all populations (Fig. 49).

These results suggest that the deletion of the L2a element leads to increased numbers of both CD4 and CD8 cells. The increased CD8 cell number may be the result of deletion of a potential CD8 enhancer, whereas the increased CD4 cell population may indicate more complicated, cell non-autonomous effects in the absence of L2a element. Considering that two of the KI-ΔL2a mice showed no changes, the phenotypes of these mice may be affected by subtle differences or some type of variegation among individual mice and variations among mouse strains (C57BL/6 vs 129Sv). More backcrosses to C57BL/6 are needed to obtain a pure background for establishing a more consistent phenotype.

Thymocytes from KI-ΔL2a mice were tested for T cell developmental markers CD3, CD5 and CD69 (Fig. 50). The expression levels of these surface makers were not affected, except for modest changes in CD3 levels.

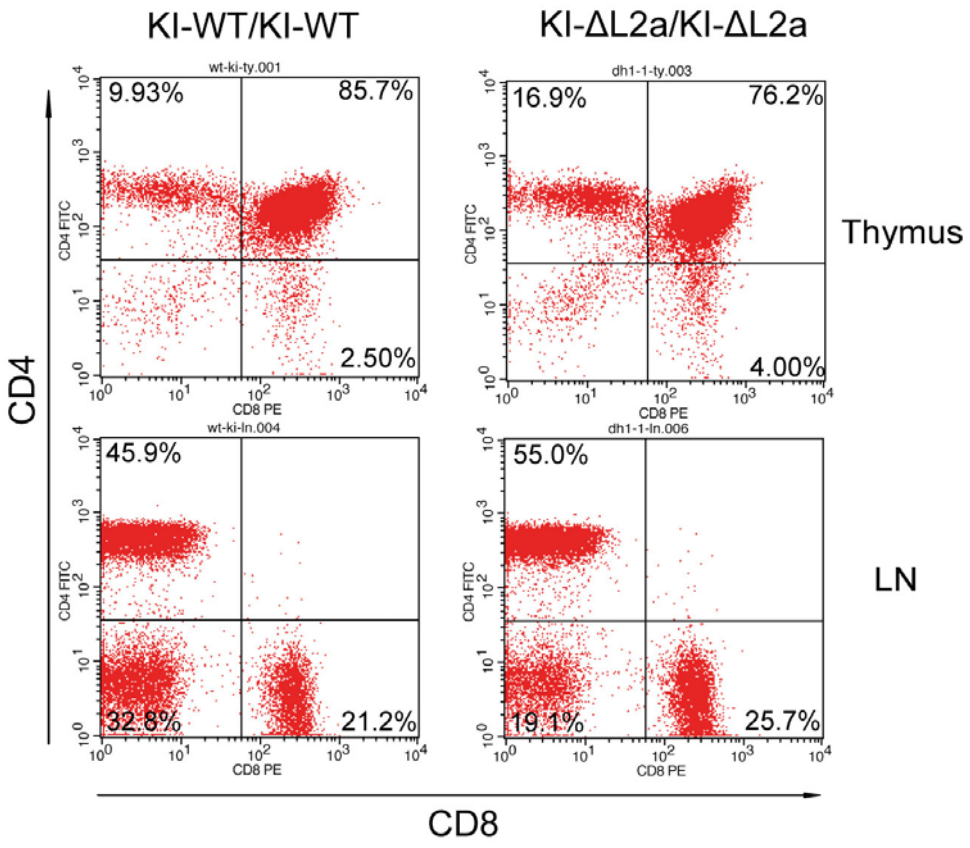


Fig. 49. KI-ΔL2a mice show modest changes in CD8 and CD4 expression.
Lymph node cells and thymocytes were isolated from homozygous KI-WT and KI-ΔL2a mice and stained with antibodies against CD4 and CD8α molecules. The cells were analyzed by FACS. Percentages of each cell population are shown. Three out of five KI-ΔL2a homozygous mice showed changes in both CD4 and CD8 expression in thymocytes and lymph node T cells.

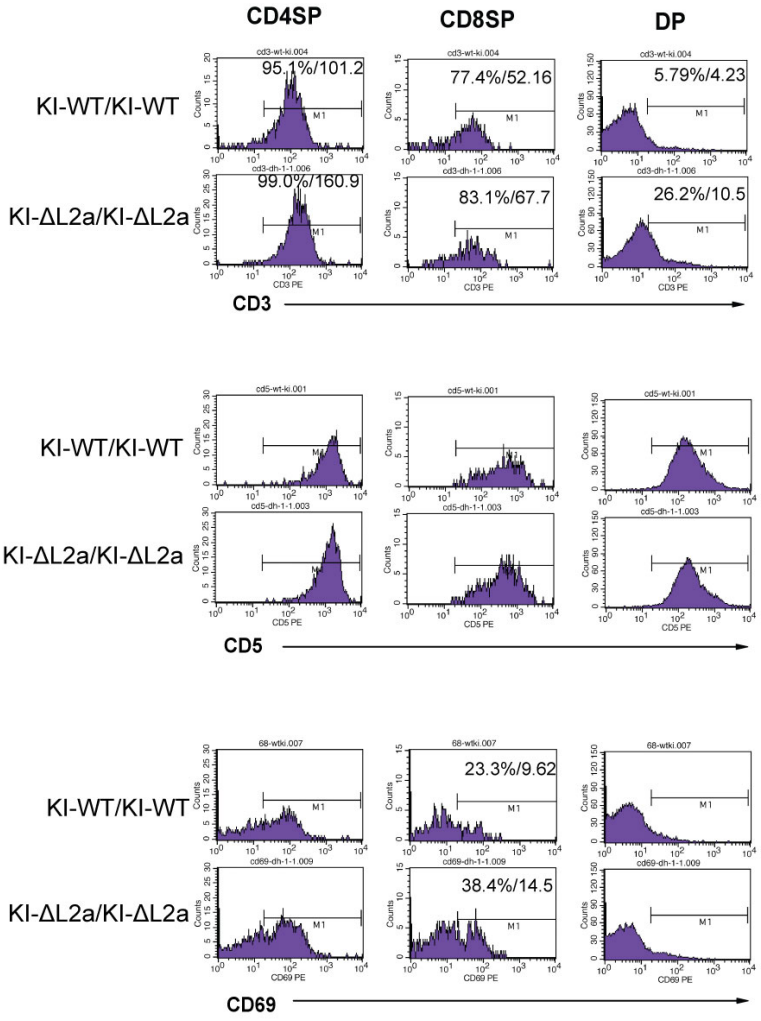


Fig. 50. Expression of T cell surface markers is unaffected in ΔL2a mice.
The indicated developmental T cell surface markers were tested on CD4SP, CD8SP and DP thymocytes. KI-WT and ΔL2a mice showed similar expression levels of CD3, CD5, and CD69 molecules. Modest changes in percentages and MFIs of positive cells are shown.

3.3.4 CD8αα and CD8αβ expression is modestly increased in IELs of KI mice

L2a KI mice were tested for expression of CD8αα homodimers in their IELs. As shown in Fig. 51, modestly increased CD8αα expression (both percentage and MFI) was observed on both TCRαβ$^+$ and TCRγδ$^+$ IELs from KI-M1 mice. CD8αβ levels on TCRαβ IELs increased as well. The KI-ΔL2a mice showed similar changes in all three IEL subsets tested (Fig. 52). Although the observed increase in CD8 expression was modest, both KI-M1 and KI-ΔL2a mice showed a consistent trend toward greater CD8 expression on their IELs. These changes suggest that both M1 and L2a deletion mutations affect the function of L2a as silencer.

3.3.5 The L2a element collaborates with $E8_I$ to promote CD8αα expression in activated lymphocytes

The CD8αα molecules induced upon antigenic stimulation promote the survival and differentiation of activated lymphocytes into memory CD8 T cells, and this process is controlled by the $E8_I$ enhancer [4]. The L2a element and DH cluster II region have been shown to collaborate with $E8_I$ in transgenic studies [5]**.** Thus, it would be informative to determine whether L2a is involved in the induced expression of CD8αα in activated lymphocytes.

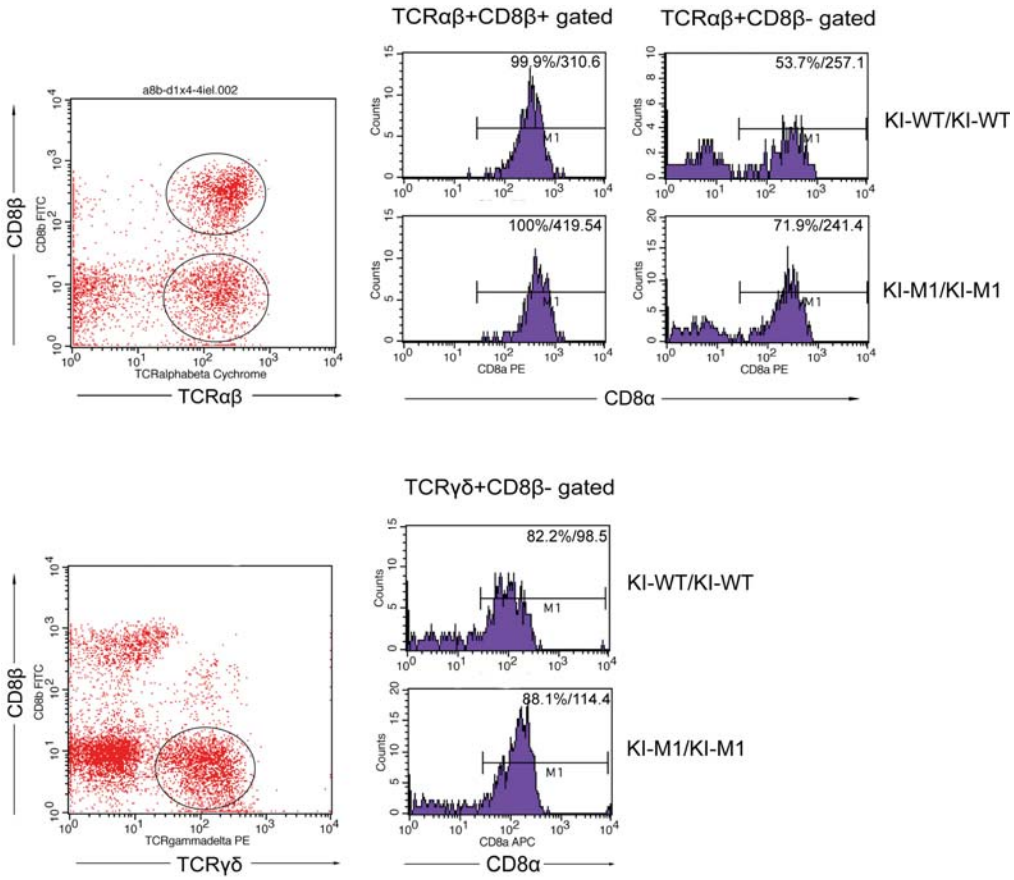


Fig. 51. CD8αα expression is modestly increased on IELs from KI-M1 mice.
Expression of CD8αα homodimers on IELs was tested in KI-WT and KI-M1 mice. IELs were stained, gated, and analyzed as shown above. Percentages and MFIs of positive cells are shown.

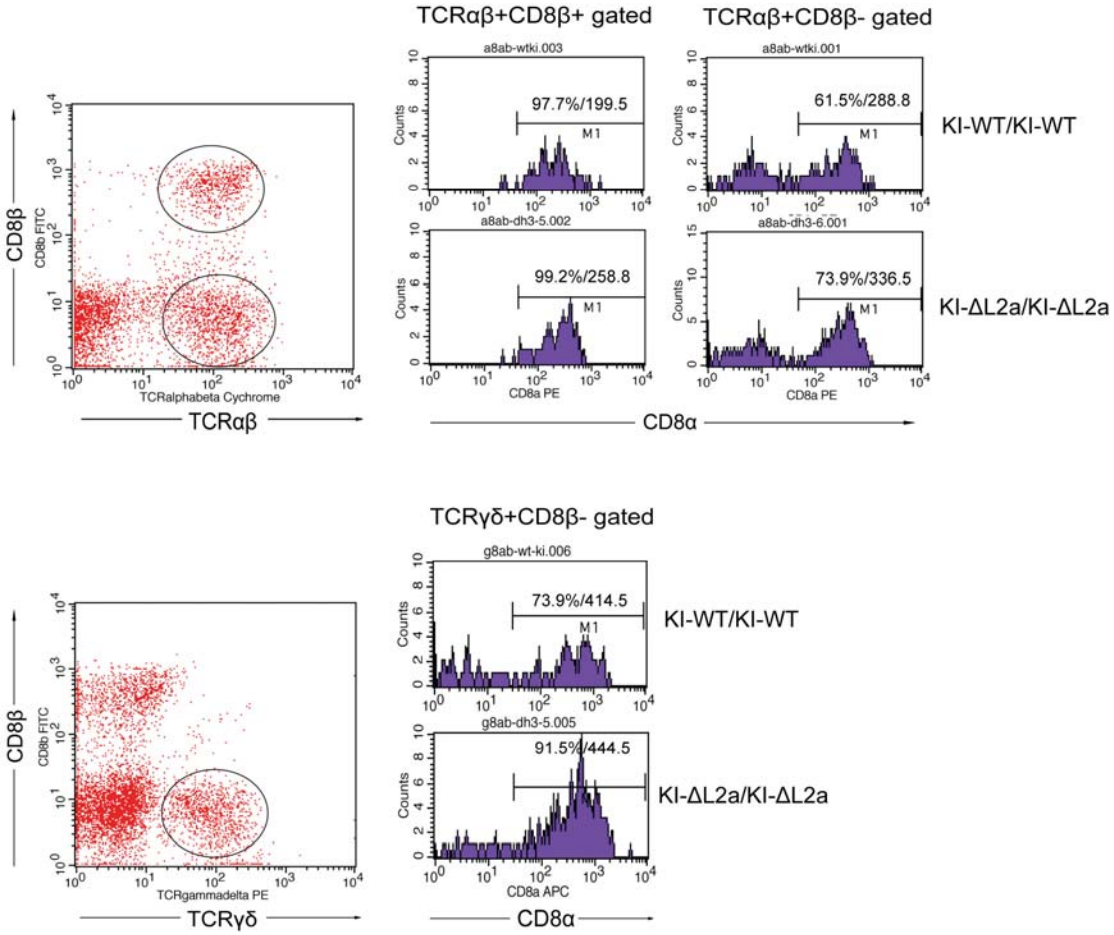


Fig. 52. CD8αα expression is modestly increased on IELs from KI-ΔL2a mice.
Expression of CD8αα homodimers in IELs was tested on KI-WT and KI-ΔL2a mice. IELs were stained, gated, and analyzed as shown above. Percentages and MFIs of positive cells are shown. In both KI-M1 (Fig. 51) and KI-ΔL2a mice, CD8αα homodimers on TCRαβ$^+$ or TCRγδ$^+$ IELs and CD8αβ heterodimers on TCRαβ$^+$ IELs showed a trend toward higher CD8 expression in L2a-deleted mice.

Splenocytes from KI-WT, KI-M1 and KI-ΔL2a homozygous mice were cultured and stimulated with anti-CD3ε antibody. CD8αα expression was analyzed by staining with thymic leukemia (TL) tetramers (kindly provided by Dr. Hilde Cheroutre, La Jolla Institute of Immunology) after 4 days of culture. As shown in Fig. 53, activated splenocytes from KI-ΔL2a mice showed increased CD8αα expression upon stimulation. This increase in CD8αα is consistent with the hypothesis that L2a is a silencer, and its deletion has relieved repression of TCR-mediated induction of CD8αα expression—an event critical to survival and memory cell differentiation.

3.3.6 Decreased CD8αα expression in dendrite cells of KI-ΔL2a mice

The expression of CD8αα homodimers in a subset of DCs was analyzed as shown in Figs. 54 and 55. The relative frequency of $CD11c^+$ DCs from KI-M1 mice that expressed CD8αα (27.4%) was indistinguishable from that of KI-WT mice (20.4% and 27.5%). However, the frequency of CD8αα expressing DCs from KI-ΔL2a mice decreased significantly (8.8%). These data strongly suggest that the L2a element may function differently in the development of $CD8\alpha\alpha^+$ DCs, which belong to a different cell lineage.

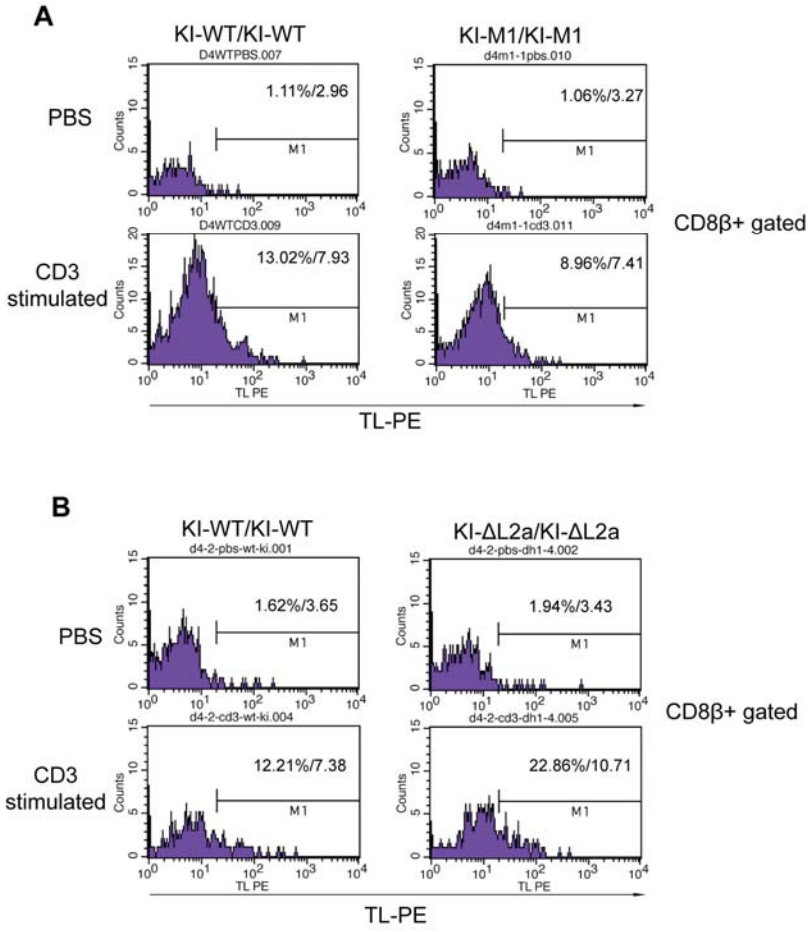


Fig. 53. L2a collaborates with E8$_I$ for CD8αα expression in activated peripheral T cells.

Splenocytes from KI-WT, KI-M1 and KI-ΔL2a homozygous mice were cultured and stimulated with anti-CD3ε antibody, and CD8αα expression was analyzed by staining with thymic leukemia (TL) tetramers on day 4. Percentages and MFIs of positive cells are shown within each profile box. Activated splenocytes from KI-ΔL2a mice showed increased CD8αα expression upon stimulation.

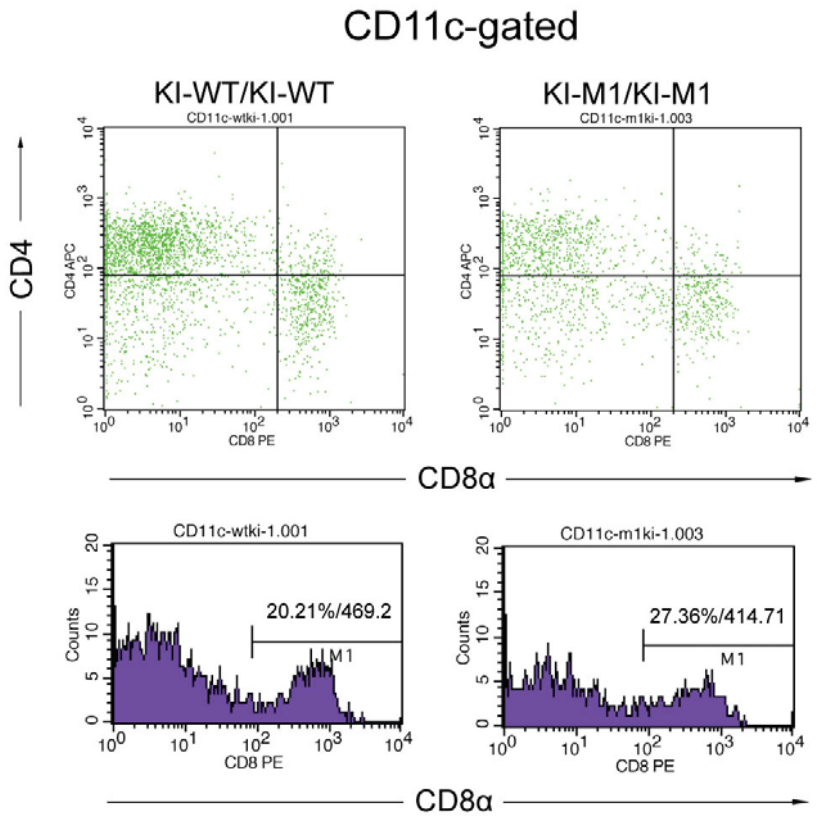


Fig. 54. Unaltered CD8αα expression on dendrite cells of KI-M1 mice.
The expression of CD8αα homodimers in a subset of DCs was analyzed as shown in previous FACS figures. Percentages and MFIs of positive cells are shown. $CD11c^{+}$ DCs from KI-M1 mice expressed the same level of CD8αα (27.4%) as those [Fig 54] of KI-WT mice (20.4% and 27.5%).

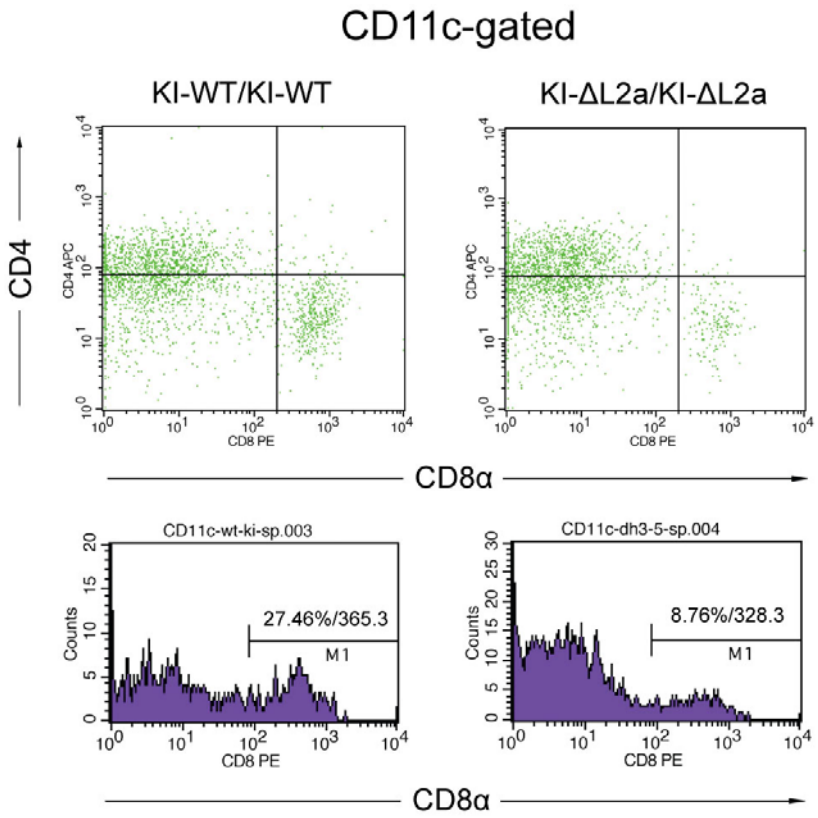


Fig. 55. Decreased CD8αα expression on dendrite cells of KI-ΔL2a mice.

The expression of CD8αα homodimers in a subset of DCs was analyzed as shown above. 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.

4. DISCUSSION

4.1 The L2a MAR, SATB1 and CDP/Cux and the Displacement Switch Model

Nuclear matrix-associated regions (MARs) are short AT-rich DNA sequences that are widespread throughout the eukaryotic genome, and have great affinity for the nuclear matrix *in vitro* [6, 7]. MARs have been postulated to mediate chromatin loop formation important for compaction of genomic DNA, and also to organize chromatin into units of genomic function [8, 9]. A stable transfection approach was used to define an ~200 bp *cis*-acting element (L2a) 4.5 kb upstream of *CD8a* that appeared to be the target of negative regulation in hybridomas produced by fusion of $CD8^+$ cells with BW5147 [10]**.** Prompted by the AT-rich nature of L2a, it was tested and confirmed to be a MAR using the conventional *in vitro* matrix binding assay.

Several MAR-binding proteins have been identified, and two of these, SATB1 and CDP/Cux were shown to specifically interact with L2a [3]. Footprinting analysis demonstrated that two protected regions (L and S) are the binding sites of CDP/Cux and SATB1 [3]. On the basis of earlier studies of the cell type distributions of SATB1 and CDP/Cux and their interaction with the L2a element [3, 10], it was proposed that SATB1 and CDP/Cux play positive and negative roles, respectively, in *CD8a* gene regulation [3]. They suggested that binding of SATB1 displaces the CDP/Cux repressor from the L2a element and favors CD8α expression, probably by altering chromatin structure and/or allowing the L2a element and nearby *CD8a* gene to associate with the nuclear matrix [3]. This proposed model describing the interaction of SATB1 and CDP/Cux with the L2a element was termed as the Displacement Switch Model (Fig. 5).

The CDP/Cux protein contains a homeodomain and three cut repeats, and each of them may have specific, unique DNA binding activities [11, 12]. The results of EMSAs performed here with elongated L2a probes indicated that both the L and S regions are important for CDP/Cux binding (Figs. 8-12). Binding of CDP/Cux to the GC-4 probe was decreased dramatically but was not completely lost. This suggests that individual binding of CDP/Cux to the L or S region is not strong enough to maintain the interaction. Thus, DNA motifs that contain two binding sites separated by an appropriate distance may be the preferred target of CDP/Cux. Two binding sites may provide more options for other regulatory factors, such as SATB1, to compete for associated regions. This may provide the basis for the Displacement Switch Model.

The interaction between SATB1 and elongated L2a probes yielded a supershifted binding pattern (Figs.13-16). Footprinting experiments indicated that the binding of CDP/Cux to L2a probes did not change the DNA conformation, whereas the interaction between SATB1 and L2a probes caused a dramatic increase in the DNase I hypersensitivity (Fig. 17). This implicated SATB1 as a positive regulator.

In addition to the L2a element-associated SATB1 site, SATB1 binding sites were identified within DH cluster II and within the CD8 enhancer $E8_{III}$ (Figs. 26-28). Interestingly, all the SATB1 sites identified in this report overlap with previously established [13] DH sites, suggesting that SATB1 is important for the chromatin remodeling process. This, coupled with the previously observed defects in CD8 expression under conditions of SATB1 deficiency [14, 15], suggests that SATB1 binding sites may be good candidates for additional DNA *cis*-acting elements critical to CD8 regulation.

CDP/Cux was also found to bind to multiple regions within DH cluster II and within the $E8_{III}$ enhancer (Figs. 26-31), but only a few of them showed

strong interaction. Most of the complexes are relatively weak, suggesting they are not very specific or that they are only partially qualified for CDP/Cux binding according to the criterion discussed above. As for SATB1, fewer binding sites were found, and surprisingly, each SATB1 binding site identified was also a strong binding site for CDP/Cux. It appears that these two proteins work together as partners, since their binding sites are always, at least partially, overlapped. Similar observations were made by other groups [16, 17]. Thus, the Displacement Switch Model might have a more general basis and could be used to predict the consequences of the interactions among SATB1, CDP/Cux and their binding sites.

Unpublished experiments from Ingrid Rojas (personal communication) using protein competition assays with SATB1 and CDP/Cux demonstrated that SATB1 binds predominantly to the L2a element. When bound together to L2a, SATB1 and CDP/Cux proteins did not interfere with each other, nor did they coimmunoprecipitate in VL3 and Jurkat nuclear extracts. These observations provide further evidence in support of the Displacement Switch Model.

4.2 New *cis*-acting elements involved in CD8 expression

The palindromic 12-mer in the INTER-LS region of L2a may be another regulatory sequence involved in CD8 expression. Introduction of one PAL insert into the INTER-LS region completely altered the binding patterns of SATB1 and CDP/Cux (Fig. 14 and 20). Two point mutations within the 12-mer weakened CDP/Cux binding and abolished complexes (termed A and B) formed by unidentified proteins (Fig. 19). These observations indicated that the palindromic 12-mer may be involved in the interaction of L2a with SATB1 and CDP/Cux. To do this, additional 12-mer binding factors might be recruited to this region.

Two 12-mer binding proteins were identified by affinity purification. One of these, pigpen, modulates endothelial cell differentiation [18], while the other is an EST without known function. Both have DNA binding domains, and further functional study is required to determine their role in the context of the palindromic 12-mer. In addition, the OAZ family of proteins was found to have a preferred DNA binding site that is almost identical to the 12-mer [19]. Since OAZ proteins contain multiple DNA and protein interaction domains and are involved in several important biological functions [20, 21], it will be interesting to test if they can bind to the 12-mer and regulate CD8 expression.

Our transgenic studies indicated that the L2a element might be a silencer of CD8 expression. The DH cluster II fragment without L2a drove E8I to activate a CD8 reporter in DP thymocytes, suggesting that other *cis*-acting elements in this region may be responsible (Fig. 33-36). We identified two strong SATB1 binding sites proximal to the second DH site of cluster II (CII-2) (Fig. 26). These might be good candidates for *cis*-acting elements that collaborate with the E8I enhancer. The results here and those of others indicate that the DH cluster II may be a complicated region, comprised of the putative L2a silencer and other positive regulatory elements.

Both the E8III enhancer [22] and the SATB1 protein (H. Nie, personal communication) have been shown to be involved in the corecepter reversal process during positive selection of thymocytes. We identified a strong SATB1 binding site near the 5' end of the E8III enhancer (contained within a 1.5 kb SacI/BamHI fragment) (Fig. 29), which might be the target of the SATB1-mediated effect. Since the 285 bp core portion of the E8III enhancer resides at the 3' end of that fragment [23], E8III may also be a combination of enhancer and other *cis*-acting DNA elements.

4.3 The L2a element as a CD8 silencer

Transgenic studies reported here indicated that the L2a element is a CD8 silencer. In mice containing the L2a wild type transgene (L2aWT), CD8 reporter (hCD2) expression is silenced in both DP and CD8SP thymocytes (Fig. 33 and 34), suggesting that the L2a silencing may occur at an early developmental stage, such as DN. A small portion (~5%) of CD8SP thymocytes and CD8 T cells escaped silencing, resulting in variegated reporter expression within these two populations. When the L2a element was deleted (L2aD mice), significant expression of the CD8 reporter was observed in all CD8 expressing cells, including DP thymocytes (Fig. 35 and 36).

CD8 reporter expression on the cell surfaces of CD8αβ and CD8αα positive IELs from both L2aWT and L2aD mice were indistinguishable from the patterns observed on mature CD8 T cells (Fig. 38 and 39). We also detected "escaped" variegated reporter expression in these populations. These results suggest that CD8 expression in IELs is control by L2a silencing and that thymocytes and IELs may share a similar CD8 regulatory mechanism.

When the L2WT mice were crossed onto a SATB1-deficient background, the small fraction of "escaped" variegated-expressing CD8SP thymocytes and CD8 T cells was reduced (Fig. 42). This suggests that SATB1 is involved in re-starting silenced CD8 expression mediated by L2a. That SATB1 might overcome L2a silencing to re-express CD8 at certain developmental stage is consistent with unpublished results from our laboratory (H. Nie, personal communication). Her analysis of SATB1-null mice indicated that SATB1 is indispensable for re-initiation of CD8 transcription during the coreceptor reversal process (transition from $CD4^{+}CD8^{low}$ to CD8SP cells) during positive selection.

All the results from transgenic studies suggest that the L2a element is a silencer involved in CD8 expression, which is consistent with the negative regulatory role of L2a reported in previous results [10] and cell transfection results using L2a construct (Fig. 24 and 25). The L2a silencer may play an important role in the CD8 expression which is controlled by multiple enhancers and redundant regulation.

4.4 Knock-in studies on the L2a element

We performed knock-in studies to further investigate the function of L2a. The M1 mutant knock-in mice, which have altered binding sites in the L2a element that abolish SATB1 interaction, showed no significant change in T cell development or in CD8 expression in thymocyte or in peripheral T cell subsets (Fig. 47 and 48). Three out of five knock-in mice (KI-ΔL2a) in which the entire L2a element was deleted had modestly increased numbers of CD8 thymocyte and CD8 peripheral T cell populations (Fig. 49). This result is consistent with a potential silencer function for L2a. IELs form both knock-in mice showed a trend of modestly increased CD8α expression in CD8αβ and CD8αα cells (Fig. 51 and 52), further indicating that loss of SATB1 binding to the L2a element contributes to silencing CD8 in IELs.

All the modestly increased CD8 expression observed in thymocytes, peripheral T cells, IELs and activated lymphocytes (Fig. 47-53) indicates the consequence of the deleted L2a silencer. Compared to the more dramatic silencing observed in transgenic analyses, it is possible that compensatory effects of other *cis*-acting elements present in the germline configuration but missing from the transgenic locus may result in less significant effects observed in L2a mutant knock-in studies.

4.5 Function of L2a in dendritic cells

The murine DCs have been classified into two lineages, lymphoid DCs [24] and myeloid DCs [25]. Based on expression of CD4 and CD8αα homodimer, which were thought to be expressed mainly on T cells, DCs can be further divided into different subtypes [26]. CD8αα$^+$ DCs, which lack the myeloid maker CD11b, were originally thought to develop from lymphoid-committed thymic T cell progenitors at low frequencies [27]. CD8αα$^-$ DCs, which are either CD4$^+$ or CD4$^-$ and generally express CD11b, were thought to arise from myeloid progenitors [28]. However, Traver *et al.* demonstrated that both CD8αα$^+$ and CD8αα$^-$ DCs are generated from common myeloid and lymphoid progenitors in both mouse thymus and spleen [29]. Their results suggest that CD8αα on DCs reflects the differentiation or maturation status of DCs but does not indicate a lymphoid origin [29].

In our knock-in studies, CD8αα expression on the CD11c$^+$ DCs (CD11c is a common maker for all DCs) was significantly decreased in KI-ΔL2a mice (Fig. 55). Unlike the function of a silencer in T cells and IELs, L2a may play a different role in the regulation of CD8αα expression in DCs. Furthermore, in L2a transgenic mice, a subset of CD11c$^+$CD8αα$^+$ DCs was shown to express the reporter at a much lower level than CD8 T cells (Fig. 40), suggesting that the regulation of CD8α expression in DCs is different from that of T cells.

Both knock-in and transgenic studies suggest a possible different regulatory mechanism of CD8 expression between DCs and T cells. This indicates that the CD8αα$^+$ DCs may not be, at least partially, derived from lymphoid lineage. The L2a element, which has been shown to be a silencer in T cells of lymphoid lineage, may play a different role in CD8αα expression in DCs (possibly from a myeloid origin).

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