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Pre-TCR Signaling and CD8 Gene Bivalent Chromatin Resolution during Thymocyte Development

Nicola Harker,* Anna Garefalaki,* Ursula Menzel,† Eleni Ktistaki,* Taku Naito,‡ Katia Georgopoulos,* and Dimitris Kioussis*

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E expression of coreceptors CD4 and CD8 mark the differentiation stages of thymocyte development. CD4−CD8− T cell precursors in the thymus start expressing the pre-TCR complex, which enables them to pass the β selection checkpoint. Subsequently, the α- and β-chains of TCR and CD4 and CD8 are expressed to generate CD4+CD8+ double-positive (DP) cells that undergo positive and negative selection to ensure that only fully functional and harmless T cells are released in the periphery (reviewed in Ref. 1). On most CD8+ cells, the coreceptor is expressed as a disulphide-linked CD8α/CD8β heterodimer (2–4). The CD8α and CD8β genes are located on chromosome 6 (5), and all of the sequences required for full and appropriate expression of the CD8αβ heterodimer reside within an 80-kb genomic fragment (6). There are a growing number of proteins regulating the CD8 locus, with Ikaros, Runx1, Baf57, and Brg implicated in the activation of transcription (7–10) and MAZR being associated with CD8 repression (11). Differentiating cells activate or silence genes implicating chromatin-remodeling processes, such as histone modifications and nucleosome displacement (12–19).

Histone modifications include trimethylation of lysine 4 of histone H3 (H3K4me3), associated with a transcriptionally permissive status (20, 21) and trimethylation of lysine 27 of histone H3 (H3K27me3) associated with repression (22). These two modifications, until recently, were deemed to be mutually exclusive. However, work by Azaoua et al. (23) and Bernstein et al. (24) showed that these two marks can coexist at the promoter of a number of low or nonexpressing gene loci in mouse embryonic stem (ES) cells. This phenomenon is described as bivalency and the regions of the chromosome exhibiting these marks as bivalent chromatin. Such bivalent states were also identified at the promoter of genes of more differentiated cell types, such as naive Th cells, CD8+ memory T cells, and macrophages (25–27). Bivalency is currently considered to be an important control mechanism by which a gene locus acquires a poised configuration and is thought to be resolved in subsequent stages by the loss of one and the retention of the other of these two modifications, leading to full activation or silencing, accordingly.

In recent years, epigenetic modifications of chromatin during thymocyte development and lineage commitment have been described (9, 11, 28–31). However, it is unclear at the moment whether the mechanisms governing bivalent chromatin also apply at branching fate decision points of thymocyte differentiation (32). To address this issue, we have undertaken a kinetic DNaseI hypersensitive site analysis of the murine CD8 gene complex, as well as an assessment of its histone code and association with chromatin remodeling activities during thymocyte differentiation.

In this study, we show that partial remodeling of the CD8 locus is apparent at the double-negative (DN) 3 stage before CD8 expression. Interestingly, pre-TCR signaling at the β selection point is followed by full accessibility of the locus, whereas commitment to the CD4 lineage results in its gradual inaccessibility. Importantly, histone code analysis of the locus reveals bivalent characteristics of negative and positive marks at the DN stage, right before the locus becomes activated. We provide evidence that Ikaros associates with the locus throughout all the stages of development and that in nonexpressing cells, Ikaros plays a role in the regulation of the locus, probably either by recruiting on-site Mi-2β, a component of the negative chromatin remodeler NuRD, or by antagonizing Mi-2β.

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Abbreviations used in this article: ChIP, chromatin immunoprecipitation; Ct, cycle threshold; DN, double-negative; DP, double-positive; ES, embryonic stem; H3K27me3, trimethylation of lysine 27 of histone H3; H3K4me3, trimethylation of lysine 4 of histone H3; HP1α, heterochromatin protein 1α; IP, immunoprecipitation; NIMR, National Institute for Medical Research; qPCR, quantitative PCR; S2, Schneider 2; SP, single-positive.
Materials and Methods

**Mice and cell culture**

C57BL/10 (National Institute for Medical Research [NIMR]), C57BL/6 (NIMR), and B6/J Rag1<sup>−/−</sup> MOM mice (33) were housed in specific pathogen-free conditions and euthanized via a schedule 1 procedure via exposure to CO<sub>2</sub>. The authors confirm that all experiments were performed in accordance with the guidelines and regulations of the Home Office UK and in accordance with the review panel of the Medical Research Council. Drosophila melanogaster Schneider 2 (S2) cells were grown at 28˚C without CO₂ in Schneider’s Drosophila Medium (Invitrogen) with 10% heat-inactivated FBS (Bio RSA).

**Cell sorting**

For the isolation of wild-type T cell populations, thymus and spleen were taken from 20 C57BL/6 or C57BL/10 mice and stained with the following Abs. Peripheral CD4 and CD8 single-positive (SP) T cells were stained with anti-TCR<sub>γ</sub> FITC, anti-CD4 PE, and anti-CD8 allophycocyanin; CD4 SP and CD8 SP thymocytes were stained with anti-CD4 allophycocyanin, anti-CD8 FITC, and anti-CD24 PE, and the cells were sorted as CD<sup>+</sup>/CD24<sup>−</sup> and CD8<sup>+</sup>/CD24<sup>−</sup>; DP thymocytes were stained with anti-CD8 FITC and anti-CD4 allophycocyanin; B cells were stained with anti-CD19-PE (BD Pharmingen). The cells were sorted using the Cytometry MoFlo high-speed cell sorter (DakoCytomation).

**DNaseI analysis**

Nuclei from C57BL/10 and B6/J Rag1<sup>−/−</sup> MOM mice were prepared using the method by Forrester and colleagues with adaptations (34). A total of 1 × 10<sup>7</sup> cells/sample were lysed in 0.5% Nonidet P-40/RSB and incubated with 1 µl CaCl<sub>2</sub> and increasing concentrations of DNaseI (0, 20, 40, and 80 ng; Sigma-Aldrich) at 37˚C for 4 min. DNaseI activity was inhibited with 100 µg/ml DNaseI stop mix (0.6 M NaCl, 20 mM Tris [pH 8], 10 mM EDTA, and 1% SDS). Following a proteinase K step, DNA was extracted by phenol/chloroform extraction and ethanol precipitated. A total of 100 ng DNA was mixed with 1 µl of [γ<sup>32</sup>P]deoxycytidine triphosphate (GE Healthcare) by Southern blot and hybridized with an mCD8<sup>+</sup> RNA, the RiboQuant RPA kit (BD Pharmingen), and the mCD-1 Multi-Probe template set (BD Pharmingen). Probes were labeled with 10<sup>6</sup> T C<img class="capsule" data-capsule-type="superscript" data-capsule-value="g"/>y-activated T cell (IgG), and the resultant product was quantified by incorporation of SYBR Green (Applied Biosystems) on Applied Biosystems 7900HT. Cycle threshold (C<sub>T</sub>) values for each sample were normalized to a nonhypersensitive region of the locus (Exon V), whereas any change in product with increasing DNaseI concentration was expressed as a percentage of DNaseI untreated sample.

**Chromatin immunoprecipitation analysis**

Chromatin immunoprecipitation (ChIP) analysis of the CD8 locus was undertaken in accordance with the Upstate Biotechnology protocol, and all results shown are representative of at least three independent experiments. In brief, CD4 SP, CD8 SP, DP, or DN cells were obtained from C57BL/6 mice and FACS sorting was performed. A total of 1 × 10<sup>7</sup> cells/chromatin immunoprecipitation (IP) were used for all cell populations except the DN subpopulations, in which for each IP, 1 × 10<sup>5</sup> DN cells were supplemented with carrier chromatin from 1 × 10<sup>5</sup> Drosophila melanogaster S2 cells (35). Cells were subjected to formaldehyde cross-linking at 37˚C for 10 min (1% formaldehyde), and cross-linking was stopped with sodium hydroxide. Samples were sonicated to a mean size of 100 bps and immunoprecipitated using the ChIP from C57BL/10 (National Institute for Medical Research [NIMR]), C57BL/6 (NIMR), and samples were sonicated using Bioruptor UCDO-200 (Diagenode). The size of sonicated DNA was checked to be between 200–500 bp on 1% agarose gel, and a 50-µl sample was reserved as input. Samples were diluted and preclarified with protein A or protein G agarose beads and incubated with Abs overnight at 4˚C with rotation. Chromatin was immunoprecipitated with the following ChIP-grade Abs: anti-HP1α, anti-Ikaros (Katia Georgopoulos, Harvard Medical School, Boston, MA), and anti-heterochromatin protein 1α (HP1α; Millipore).

Immunoprecipitated complexes were captured with protein A or protein G agarose beads for 4 h at 4˚C with rotation, and the beads were subjected to washes of increasing stringency. DNA–protein complexes were eluted, and cross-linking was reversed with NaCl at 65˚C overnight. Samples were treated with RNase and digested with proteinase K; DNA was phenol extracted and ethanol precipitated in the presence of 10 µg glycogen. Samples were run by real-time PCR.

**Real-time PCR analysis of ChIP samples**

DNA was amplified with primers spanning the CD8-hypersensitive sites: CII-1F, 5'-GTT GAT GAT AAG CGA AGC ACT AC-3'; CII-1R, 5'-CTG CTT CAG TAT CTA GCT-3'; CII-2F, 5'-TAC TCC CTA GGC AGC ACC GAT TAG TGT GGT GCT CAT ATC-3'; CII-1R, 5'-GTA TCA CAG AAG GGC TGC AGG-3'; CII-2F, 5'-ATG TCG TAA GTT CCA GCC GGC C-3'; CII-2R, 5'-CTG TCT CCA ACT AGA ATG AAG C-3'; CII-3F (promoter), 5'-AAT TCC TCC CAC TGT CTC TGC-3'; CII-3R, 5'-CTA GCT TGA CCA AAG CTG CCT-3' (7); Exon V, 5'-GCC CTA GTC AGA CAG GAA GGC AA-3'; and Exon V, 5'-TGT GAT GGG GAC AGT TCC T-3'. Control primers used were: Oct4F, 5'-GCC TCT CCA GAG GAG GGC TGA G-3'; Oct4R, 5'-TCT CCA GCC CCA TGC CA-3' (Dr. Jacqueline Mermod, King’s College, London, U.K.). Thy1F, 5'-CTC CAA AGC CCA AAC CTG TC-3'; and Thy1R, 5'-GCT GAT GCC AGG TGG TGC AT-3' (36). DNA was quantified by incorporation of SYBR Green (Applied Biosystems) on Applied Biosystems 7900HT (Applied Biosystems). Samples were loaded in triplicate, and average C<sub>T</sub> values were obtained. The C<sub>T</sub> values were normalized both to input and binding of a mouse-specific Ab (IgG), and expressed as percent of input using the following equation: [100 x 2<sup>(C<sub>T</sub> Input − C<sub>T</sub> IP)] − [100 x 2<sup>(C<sub>T</sub> Input − C<sub>T</sub> IgG)].

**Results**

B6/J Rag1<sup>−/−</sup> MOM thymocytes exhibit a hypersensitive CD8 locus

Previously transgenic and knockout analysis had shown the importance of DNaseH Hypersensitive clusters II, III, and IV (Fig. 1A, CIII, CIV) for CD8α expression (6, 37–43). We present in this study a kinetic analysis of the appearance and maintenance of
hypersensitive sites during thymocyte differentiation. To investigate the status of the CD8 locus in DN thymocytes, DNaseI hypersensitivity analysis of thymocytes from B6/J Rag1−/− MOM (Rag1−/−) mice and sorted C57BL/10 wild-type total DN thymocytes was undertaken. Rag1−/− mice were used to take advantage of the high proportion of DN cells in this strain, which results from the arrest of T cell development at the β selection checkpoint.

Fig. 1B, panel 1, shows DNaseI sensitivity analysis of Rag1−/− thymocytes, indicating that the promoter of the CD8α locus (CII-3 site) is hypersensitive when transcription from the CD8 locus is undetectable (Supplemental Fig. 1). Analysis of total DN cells from C57BL/10 mice (Fig. 1B, panel 2), including post-β selection DN4 stage cells, shows that, in addition to CII-3 site, sites CII-1 and CII-2 are also detected. These data suggest that signaling through the pre-TCR results in the full remodeling of the locus before the onset of CD8 expression. In some of the cell populations, the DNaseI hypersensitivity sites are difficult to visualize on the autoradiograph, as only a small proportion of the cells have opened the CD8 locus. Thus, the DNase I hypersensitivity site bands were quantified using Quantity One software (Bio-Rad), the results of which are shown in Supplemental Fig. 2.

The CD8 locus becomes hypersensitive to DNaseI at the DN3 stage

These findings were confirmed and refined using quantitative PCR (qPCR)-DNaseI assays (Fig. 2). Use of the qPCR technique enabled thorough analysis of DN subpopulations that would otherwise be too few in number to visualize using the Southern blotting method. In addition, this enabled wild-type thymocytes to be used, ruling out any possibility of aberrant results that could be attributed to the Rag1−/− phenotype rather than the particular cell population.

DN subpopulations from B6 mice were supplemented with Drosophila melanogaster S2 cells to prevent overdigestion with DNaseI, and the assay was performed as before. DNA was amplified using primers specific for the CD8 promoter and a DNaseI-insensitive region of the gene (Exon V). The graph in Fig. 2 shows that hypersensitivity is observed at the promoter of CD8α in DN3, DN4, and total thymocyte cell populations, but the DN1+2 cell population is insensitive to DNaseI digestion. Thus, the qPCR results confirm the data from the Rag1−/− Southern method and extended this analysis by showing that the CD8 locus begins to open from the DN3 stage onwards and that prior to this the locus is fully closed.

FIGURE 1. DNaseI hypersensitivity site assay of differentiating T cells. A, Figure showing the CD8 gene complex, with the CD8β gene 32 kb upstream of the CD8α gene, the three CD8-specific clusters of hypersensitive sites (6), and five CD8 enhancers (40, 43). B, DNaseI hypersensitivity site assay of T cell populations from the thymus and periphery of C57BL/10 mice (DN1–3 cells from B6/J Rag1−/− MOM mice) showing the appearance of CII-hypersensitive sites with increasing DNaseI concentration (0, 20, 40, and 80 ng DNaseI). The position of the hypersensitive sites CII-1, CII-2, and CII-3 are marked along with the parent bands (indicated by *). The accompanying schematic shows the developmental stages of T cell development. This figure is representative of at least three independent sorting and DNaseI experiments.
DNaseI analysis of Rag1−/− thymocytes forced through β selection

To determine whether pre-TCR signaling induces the full opening of the CD8 gene locus, Rag1−/− mice were injected i.p. with anti-CD3ε (2C11). Such treatment forces Rag1−/− DN3 thymocytes through β selection and enables them to differentiate to DN4 and subsequently to DP cells (44).

Fig. 3 shows FACS (Fig. 3A) and DNasel (Fig. 3B) analyses of thymuses of Rag1−/− mice injected with either anti-CD3ε (2C11) or PBS 36 h previously. In mice injected with PBS, thymocytes are blocked at the DN3 stage and only possess a hypersensitive CD8α promoter (CII-3). In contrast, in mice injected with 2C11, a proportion of thymocytes have progressed to the DN4 stage, and the CD8 locus exhibits hypersensitivity in all three CII sites. These results support the notion that pre-TCR signaling results in the remodeling of the regulatory elements CII-1 and CII-2 from a closed inaccessible chromatin structure to an open configuration that allows transcription to occur.

CII-hypersensitive sites during differentiation toward the CD8 SP or CD4 SP lineages

To examine the hypersensitivity of CII during differentiation toward CD8 and CD4 lineages, thymocytes and peripheral T cells from C57BL/10 mice were sorted to >96% purity, subjected to DNasel digestion, and analyzed as described (Fig. 1B, panels 4 and 6). With increasing levels of DNasel, thymocytes that have just committed to the CD8 lineage (CD8+TCRhiCD24−) exhibit all three CII-hypersensitive sites, similar to DP cells (total thymocytes). However, as cells mature and enter the periphery, the CII-2 site is lost, extending previous results (38).
Fig. 1B, panel 5, shows that in CD4+ thymocytes, all three CII-hypersensitive sites are still detectable, albeit at lower intensity than in DP, despite the cessation of CD8 expression. In contrast, in peripheral CD4+ cells (Fig. 1B, panel 7), these hypersensitive sites are undetectable. Thus, it appears that there is a time lag between cessation of CD8 expression and heterochromatinization of the locus, similar to what was seen in the CD4 locus (45).

The H3/H4 tetramer is maintained in the CD8 locus during differentiation, but chromatin in DN cells exhibits bivalency

To gain more detailed information about the chromatin of the CD8 locus during differentiation, ChIP analysis was undertaken. Fig. 4 shows that H3 association with CII and the CD8α gene-body is high and constant as differentiation progresses, indicating that gross nucleosomal structure of the locus is maintained during differentiation.

Activating H3K4me3 and H3K27me3 repressive marks were determined by ChIP analysis (Fig. 5, Supplemental Fig. 3). Fig. 5 shows that the H3K27me3 modification is present at high levels throughout CII and the gene body in DN1+2 and DN3 cells, drops in DN4 cells, and almost disappears in transcribing DP, rising again in cells that cease transcription of CD8 and commit to the CD4 lineage. In contrast, this modification remains low in cells that maintain CD8 expression.

Surprisingly, immunoprecipitation of chromatin with anti-H3K4me3 also showed high levels of this activation mark over an 8-kb region including CII and the CD8α gene in the non-expressing DN1+2, DN3, and DN4 cell populations (Fig. 5). In DP cells, H3K4me3 modification is restricted to the promoter and CD8α gene. Commitment to the CD4 lineage results in very low levels of H3K4me3 throughout the locus, whereas CD8 SP cells retain the high levels of H3K4me3 around the CD8α promoter and gene body. The presence of these modifications at the promoters of Oct4 and Thy1 served as positive and negative controls; in thymocytes, H3K27me3 is enriched at the Oct4 promoter, whereas at the Thy1 promoter, H3 is enriched in the K4me3 modification (Supplemental Fig. 4).

Thus, the DN populations exhibit both the permissive H3K4me3 and the repressive H3K27me3 marks, a phenomenon that was first described in ES cells and termed bivalency (23, 24). Interestingly, this bivalency is restricted only to DN thymocytes and is not present in B cells or CD4+ peripheral T cells, both of which carry only the suppressive mark H3K27me3 (Fig. 5).

Ikaros, NuRD, and HP1α association with the CD8 locus during T cell development

Ikaros binds the CD8 gene in DP cells and mediates activation of the locus at this stage (7). Comparison of differentiating thymocytes and more mature CD4 and CD8 lineage cells by ChIP analysis determined that Ikaros associates with CII and the gene itself throughout development (Fig. 6, left panel), with this association reaching high levels in CD4 and CD8 SP cells. Thus, Ikaros appears to associate with the CD8 locus regardless of transcriptional state. Given that Ikaros can associate with either activating or repressing remodeling complexes, it is possible that in nonexpressing cells, repression is mediated by recruiting inactivating complexes, such as NuRD, or by competing with NuRD components, such as Mi-2β (29). To address this, the association of Mi-2β with the CD8 gene locus was examined (46–49). Analysis of thymus subpopulations ascertained that Mi-2β is associated with CII in DN1+2 thymocytes (Fig. 6A, middle panel). Mi-2β association is reduced dramatically in DN3 and DN4 stages and is almost undetectable in DP and CD8 SP cells. Mi-2β is, however, restored on the locus in peripheral CD4+ T cells (Fig. 6B, middle panel).

HP1α is crucial to the formation of heterochromatin (reviewed in Ref. 50). Association of HP1α with the CD8 locus was examined in the T cell subsets (Fig. 6, right panel). Similar to Mi-2β, HP1α is associated with the locus mostly in DN1+2 cells, whereas it is absent when CD8 is transcribed and expressed at DP and CD8 SP stages. Cells that have just committed to the CD4 lineage exhibit, again, high levels of HP1α; however, these levels drop in peripheral CD4 SP cells (Fig. 6B, right panel). Another ChIP analysis showing the association between Ikaros, Mi-2β, and HP1α and T cell populations is shown in Supplemental Fig. 5.

Thus, both Mi-2β and HP1α are implicated in negative regulation of the CD8 gene locus during reversible and irreversible silencing in DN and CD4 SP cells, respectively. Thus, both proteins seem to assist in silencing the bivalent locus in DN cells, and
both are removed prior to the locus remodeling at DN3 and DN4 stages. Subsequently, HP1α is involved in initial silencing of the locus in CD4 thymocytes, whereas NuRD resumes its repressive action by being recruited to the locus in peripheral CD4 SP T cells.

Discussion

Plasticity in gene expression is a hallmark of developmental processes, and the problem of how genes oscillate between active and inactive states has been a formidable challenge in molecular biology. The CD8 gene locus offers a useful paradigm to study chromatin involvement in the regulation of a gene that exhibits a complex developmental program of expression. We and others have identified regulatory DNA sequences along the locus that regulates this developmental pattern of expression (6, 7, 37–43). The chromatin structure of these sequences during thymocyte differentiation was examined by DNaseI hypersensitivity, and it was found that the hypersensitivity and accessibility are established on the locus before the onset of CD8 transcription and are retained for a short time after the cessation of its expression in the CD4 lineage in the thymus. CD4 cells in the periphery show complete absence of hypersensitivity, indicating that the repressing regulatory mechanisms have evolved in the CD4 lineage cells that orchestrate the heterochromatinization of the locus. Taken together, these data suggest that changes in chromatin accessibility are the first and

FIGURE 5. CII exhibits bivalency in DN cells. Graphs showing the presence of the repressive modification H3K27me3 and the activatory modification H3K4me3 along CII and in the CD8α gene in FACS-sorted DN1+2, DN3, DN4, DP, CD4 SP, and CD8 SP cells of the thymus and periphery. Splenic B cells are shown as control. The position of the primer sets used to analyze CII and CD8α are depicted on the figure as colored spheres and correspond to the colored lines on the graph (primer sets used 5’ CII, CII-1, CII-2, CII-3, and Exon V1). Ct values from immunoprecipitation with H3K27me3 and H4K4me3 were normalized to Ct values from immunoprecipitation with a nonspecific anti-IgG Ab; values were then expressed as percentage of input and plotted on the graphs as shown. Results are representative of three independent experiments, another example of which is shown in Supplemental Fig. 3.
last event that occurs during the on/off sequence of CD8 gene expression. This is in agreement with other examples in the literature that suggest that chromatin accessibility is established shortly before the onset of transcription (23, 24, 51).

Our data revealed that remodeling is a gradual process and dependent on external stimuli. Thus, whereas the CD8 promoter is inaccessible in the early stages of differentiation (DN1 + DN2), the promoter shows signs of remodeling at the DN3 stage. Remarkably, the establishment of full accessibility at the rest of the regulatory elements seems to require signals from the pre-TCR. Recent data have shown that the pre-TCα dimerizes autonomously to TCRβ, acting as a crucial checkpoint to ensure the correct rearrangement and folding of TCRβ and enabling subsequent signaling through the pre-TCR (52). It could be envisaged that such signaling mechanisms could be translated to chromatin modifications.

To address what epigenetic modifications are associated with these changes, we examined the histone code on the regulatory elements and the gene itself during the different stages of development. It was shown that in CD8-expressing cells, the locus has open chromatin histone marks (H3K4me3) on a narrow region (∼2 kb) comprising the promoter and the gene. In contrast, CD4 cells establish negative histone marks throughout the locus following commitment of DP cells to this lineage. Remarkably, however, in the DN stage when the chromatin of the locus is being remodeled to become accessible, we observed the existence of both positive (H3K4me3) and negative (H3K27me3) marks along a widespread region of at least 8 kb covering the 5′ regulatory elements as well as the body of the gene. Although it has been technically impossible to ascertain that the two modifications are present on the same stretch of chromatin (24), our data are consistent with

**FIGURE 6.** Ikaros is associated with the locus throughout development, whereas negative regulators associate with the locus when CD8 is not expressed. A, Figure showing the association of Ikaros (left panel), Mi-2β (middle panel), and HP1α (right panel) with CII and the CD8 gene in DN1+2, DN3, DN4, and DP thymocytes. Ct values from immunoprecipitation with anti-Ikaros, anti-Mi-2β, and anti-HP1α Abs were normalized to IgG, expressed as percentage of input, and plotted on the graphs. B, Graphs showing the association of Ikaros (left panel), Mi-2β (middle panel), and HP1α (right panel) with CII and the CD8 gene in CD4 SP and CD8 SP cells of the thymus and periphery. CD4 SP cells are represented by green bars, and CD8 SP cells are represented by red bars. Normalization of Ct values is as described in A. The map of CII shows the position of the primers used to amplify regions of the locus, which are color coded to indicate their positions on the graphs (shown as spheres; primer sets 5′ CII, CII-1, CII-2, CII-3, and Exon V). The results are representative of three independent experiments, another example of which is shown in Supplemental Fig. 5.
a hypothesis that in the stages before commitment to active transcription, the locus acquires a poised, accessible, but non-transcribing configuration. This is characterized, on one hand, by histone marks that can attract activating remodeling complexes (possibly involving Trx group) and on the other hand by the simultaneous acquisition of the repressive modification H3K27me3 (possibly via PcG proteins) that can impose a silencing configuration. Interestingly, this bivalency appears to occupy the whole gene locus (~8 kb) and is in agreement with observations made in ES/pluripotent cells in which H3K4me3 was found to cover large areas of bivalent domains (24) as opposed to the narrow distribution seen on transcribing genes.

It is thought that bivalency in a locus resolves in a stably active or stably inactive configuration by the loss of one of the bivalent marks in subsequent differentiation stages (24). Indeed, following pre-TCR signaling and progression to the DN4, and ultimately DP stage, the CD8 locus loses the H3K27me3 mark while retaining the H3K4me3 mark on the promoter region of the gene in the DP and CD8 SP stages. However, this state is not permanent, as DP cells that commit to the CD8 lineage lose this activation mark and reacquire high levels of the H3K27me3 modification accompanied by silencing of the gene. This is analogous to what has been described by Golob et al. (53) on the brachyury locus, in which a bivalent state is resolved first into an active transcribing configuration, which turns into an inactive configuration in the subsequent developmental stages, indicating, as predicted (32), that bivalence resolution does not have to be a permanent decision. Interestingly, bivalency and its resolution were also observed on master gene loci during fate determination of differentiating CD4+ Th cells following appropriate stimulus, also indicating that poised configuration can underlie rapid responses to environmental signals (32, 54). However, bivalency is not the only mechanism employed by lymphocytes to poise transcription as observed in effector gene loci in memory CD8+ T cells (55).

It is still not clear whether the bivalency observed in this study plays the same role as in the other situations in which it has been described (56). Questions that had concerned the field are how early in development these bivalent states are achieved, how they are resolved, and how this resolution can be reversed in subsequent differentiation stages. It has been reported in genome-wide studies of ES cells that the murine CD8α locus carries the H3K27me3 modification (56, 57), whereas one of these studies also reports bivalency on the human CD8α gene in these cells. In this study, we also show that pre-TCR signaling is closely linked with the resolution of this bivalency at the DN3 to DN4 transition stage. Although bivalency seems to be a mechanism by which CD8 expression is regulated in early thymocytes, it may not be a universal mechanism, as the regulation of the CD4 gene (58) and our control genes indicate. Bivalency may provide an additional level of regulation at this stage, as transcription of the CD8 gene is controlled by enhancers, whereas CD4 is regulated by silencers. Interestingly, naive CD4 cells exhibit bivalency later in their differentiation (54); the establishment of bivalent chromatin may provide a mechanism by which gene regulation can be regulated at branch point decisions.

Through these changes, such as locus accessibility, establishment of bivalency, and its resolution after pre-TCR signaling, Ikaros is found on the CD8 locus regardless of expression status. As Ikaros can associate and recruit active (BAF) or repressive (NuRD) complexes, activation or silencing may depend on the complex it recruits to the locus. This is especially interesting in the light of the antagonistic role of Swi/Snf and NuRD reported in LPS-induced macrophages (59). Indeed, in CD8 nonexpressing cell populations, the region occupied by Ikaros is also occupied by Mi-2β, which is the major ATPase of the suppressing NuRD chromatin remodeling complex. Such dual function of a DNA-binding factor determined by the activity of the associated remodeling complexes has also been described for the Pax5 gene (60).

The outcome of Ikaros associating with the CD8 locus in nonexpressing cells is as yet unclear. Because a high proportion of Ikaros in T cells is associated with the NuRD complex (47), it is possible that Ikaros recruits the complex to the locus to repress expression of CD8. However, in light of the fact that in the CD4 gene locus Ikaros and Mi-2β antagonize each other (29) and that Ikaros is involved in the activation of the CD8 locus (7), it is also possible that Ikaros attempts to activate the locus, with NuRD and HP1α antagonizing this in DN and CD4 SP cells. It would be interesting to determine how important Ikaros is to establishing the histone code of the CD8 locus during development; unfortunately, the phenotype of Ikaros knockout mice makes their use in determining this impractical (61).

Although our study has focused on histone tail modifications and chromatin remodelers, the demethylation of CpG islands also plays an important role in DNA accessibility, transcription factor binding, and subsequent transcription of a gene. Analysis of CpG methylation in the CD8 locus determined that there was a significant decrease in methylated CpGs at CHI-1 and CHI-2 in CD8-expressing cells (11). Demethylation of CpGs will alter the accessibility of binding sites, and acting in concert with other changes in the locus, such as the reduction in methylation of H3K27 and loss of negative regulators Mi-2β and HP1, may enable binding of chromatin remodelers and transcription factors resulting in nucleosome remodeling, PolII recruitment, and subsequent transcription of the CD8 gene.

Our results taken together indicate that the appropriate silencing of the CD8 gene during thymocyte differentiation is achieved by a variety of chromatin regulatory mechanisms. Thus, in DN stages, the locus is silenced by the concerted action of either Ikaros recruiting Mi-2β or by Mi-2β antagonizing Ikaros, the presence of HP1α, and the negative histone mark of H3K27me3, while at the same time maintaining a poised configuration as indicated by the H3K4me3 mark. Full accessibility and activation of the locus is achieved by the locus after the β selection point by losing the negative histone mark and the removal of Mi-2β and HP1α from its chromatin. In the thymus, commitment to the CD8 lineage results in the binding of HP1α and re-establishment of negative histone marks. Interestingly, in the peripheral CD4 T cells, HP1α is removed, and the silencing of the CD8 gene appears to be maintained by Mi-2β.

In conclusion, the data presented in this study are consistent with a hypothesis that bivalent chromatin is a basic mechanism that underlies gene regulation at key points in thymocyte differentiation and lineage fate decisions. In addition to its role in the regulation of expression of developmental genes in pluripotent cells (embryonic stem cells, neural progenitors) (23, 24) and conferring plasticity in mature cells that need to respond rapidly to external stimuli (naive Th cells) (25), it also appears to be involved in the regulation of signature genes that are switched on and off during fate decision points of differentiating lineage committed cells.

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References:


Supplemental Figure 1

Total thymocytes  Total DN

CD3ε
CD4
CD8α
CD8β
GAPDH
Supplemental Figure 1 – Transcription from the CD8 gene locus is undetectable in DN cells

Figure showing RNase protection assay of total thymocytes and sorted total DN cells from C57Bl/10 mice. The positions of transcripts for CD3ε, CD4, CD8α and CD8β are shown as well as GAPDH loading control.
Supplemental Figure 2

Immature T cell populations

Single positive T cell populations
Supplemental Figure 2 – Quantification of Southern blot analysis of DNaseI hypersensitivity site assay of differentiating T cells

Figure 1B shows T cell populations from the thymus and periphery of C57Bl/10 mice (DN1-3 cells from B6/J Rag1⁻/⁻ MOM mice) which were submitted to DNaseI hypersensitivity site assay and run on a Southern blot. The bands corresponding to DNaseI hypersensitivity sites were quantified using Quantity One software (BioRad) the results of which were plotted on graphs as shown.
Supplemental Figure 3

The figure shows the H3 K27me3 and H3 K4me3 levels in different stages of T cell development, including DN1+2, DN3, DN4, DP, CD4 SP, CD8 SP, Thymus, Periphery, and B cells. The percent input is plotted against the levels of H3 K27me3 and H3 K4me3. The bars represent the relative expression levels at different stages.
Supplemental Figure 3– CII exhibits bivalency in DN cells

Figure 5 shows the presence of the repressive modification H3K27me3 and the activatory modification H3K4me3 along CII and in the CD8α gene in FACS sorted DN1+2, DN3, DN4, DP, CD4 SP and CD8 SP cells of the thymus and periphery. Supplemental Figure 3 depicts data from a similar independent experiment. The position of the primer sets used to analyse CII and CD8α are depicted on the figure as coloured spheres and correspond to the coloured lines on the graph (primer sets used 5’ CII, CII-1, CII-2, CII-3 and Exon V). C_{T} values from immunoprecipitation with both antibodies were corrected to immunoprecipitation with non-specific α-IgG antibody and values were then expressed as percentage of input and plotted on the graphs as shown.
Supplemental Figure 4

A  Thy1 promoter

B  Oct4 promoter
Supplemental Figure 4 – The Thy1 promoter is enriched for H3K4me3 and the Oct4 promoter is enriched for H3K27me3

A) Graph showing ChIP analysis of histone tail modifications H3K4me3 (black bars) and H3K27me3 (grey bars) at the Thy1 promoter in DN1+2, DN3, DN4 and DP thymocytes. C_T values from immunoprecipitation with H3K4me3 and H3K27me3 were expressed as percentage of input following normalisation to IgG; results are representative of at two independent experiments.

B) ChIP analysis showing the enrichment of H3K4me3 and H3K27me3 at the Oct4 promoter in DN1+2, DN3, DN4 and DP thymocytes. Results were analysed as described above.
Supplemental Figure 5

A

Ikaros

CD8

Mi-2β

DN1+2

HP1α

CD4 SP

DN3

Percent Input

CD8 SP

DN4

CD4 SP

DP

Percent Input

CD8 SP

Thymus

Periphery

B

Ikaros

Mi-2β

HP1α

CD4 SP

CD8 SP

CD8α
Supplemental Figure 5 – Ikaros is associated with the locus throughout development while negative regulators associate with the locus when CD8 is not expressed

Figure 6 shows the association of Ikaros (column 1), Mi-2β (column 2) and HP1α (column 3) with CII and the CD8 gene in A) DN1+2, DN3, DN4 and DP thymocytes and B) CD4 SP and CD8 SP cells of the thymus and periphery. This figure shows another experiment from an independent sort and ChIP experiment. CT values from immunoprecipitation with α-Ikaros, αMi-2β and α-HP1α antibodies were normalised to IgG, expressed as percentage of input and plotted on the graphs. In part B, CD4 SP cells are represented by green bars and CD8 SP cells are represented by red bars. The map of CII shows the position of the primers used to amplify regions of the locus which are colour coded to indicate their positions on the graphs (shown as spheres; primer sets 5’ CII, CII-1, CII-2, CII-3 and Exon V).