Regulation of DNA methylation dictates Cd4 expression during the development of helper and cytotoxic T cell lineages

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During development, progenitor cells with binary potential give rise to daughter cells that have distinct functions. Heritable epigenetic mechanisms then lock in gene-expression programs that define lineage identity. Regulation of the gene encoding the T cell–specific coreceptor Cd4 in helper and cytotoxic T cells exemplifies this process, with enhancer- and silencer-regulated establishment of epigenetic memory for stable gene expression and repression, respectively. Using a genetic screen, we identified the DNA-methylation machinery as essential for maintaining silencing of Cd4 in the cytotoxic lineage. Furthermore, we found a requirement for the proximal enhancer in mediating the removal of DNA-methylation marks from Cd4, which allowed stable expression of Cd4 in helper T cells. Our findings suggest that stage-specific methylation and demethylation events in Cd4 regulate its heritable expression in response to the distinct signals that dictate lineage ‘choice’ during T cell development.

During metazoan development, a series of asymmetric cell divisions results in cells with a vast number of distinct phenotypes that are maintained throughout life. With rare exceptions, such as rearrangements of gene segments encoding receptors in B cells and T cells, the genome sequence remains unchanged as cells adopt new identities. Stable commitment to a lineage requires the establishment of heritable patterns of gene expression or repression without alteration of DNA sequences, via epigenetic modifications. Despite a rapidly growing body of work describing putative epigenetic regulation, physiological models in which epigenetic modulation can be functionally delineated and tested in fully differentiated cells are rare.

One of the rare examples in which heritable gene expression has been studied in depth is T cell lineage ‘choice’. Cd4+ helper T cells and Cd8+ cytotoxic T cells develop from common progenitor cells on the basis of the specificity of their T cell antigen receptors (TCRs) for complexes of peptide and major histocompatibility complex (MHC) class II molecules and MHC class I molecules, respectively. The co-receptors Cd4 and Cd8 are critical for the development and function of these lineages, as they facilitate binding of the TCR to MHC class II (Cd4) or MHC class I (Cd8). The expression of Cd4 and Cd8 defines distinct stages of thymocyte development, during which ordered rearrangements in the loci encoding the TCR occur and serve as developmental checkpoints. Early Cd4−Cd8− double-negative (DN) progenitor cells transition through four distinct stages before upregulating their expression of Cd4 and Cd8 to enter the Cd4+Cd8+ double-positive (DP) stage of development. DP cells then test their randomly rearranged TCRs for specificity for MHC class I and MHC class II. MHC class I–specific cells stably downregulate their Cd4 expression to enter into the cytotoxic lineage, while MHC class II–specific cells lose Cd8 expression and maintain Cd4 expression during differentiation into the helper lineage.

The regulation of Cd4 expression during T cell development is an ideal setting for studying epigenetic regulation, as Cd4 exhibits heritable active and silenced states that can be maintained independently of the initiating genomic elements. Elements required for this regulation have been identified in a series of in vivo genetic studies and in vitro T cell culture assays. These include a 434–base pair (bp) cis-acting silencer (S4), located in the first intron of the Cd4 locus, and a 430-bp cis-acting proximal enhancer (E4P), located 13 kilobases (kb) upstream of the transcriptional start site (TSS). S4 is essential for the repression of Cd4 at two different stages of T cell development. First, germline deletion of S4 leads to ectopic Cd4 expression in DN cells, which indicates that it is required for reversible silencing before the DP stage of development. Second, S4 is required for silencing of Cd4 in mature Cd8+ cytotoxic cells, since germline deletion of S4 results in ectopic Cd4 expression in cells of the cytotoxic lineage. However, conditional deletion of S4 mediated by Cre recombinase in mature cells of the CD8+ lineage following their egress from...
the thymus does not affect CD4 expression even after multiple cell divisions. Similarly, in mature cytotoxic cells, Cre-mediated deletion of genes encoding members of the RUNX protein complex that binds S4 to initiate Cd4 silencing fails to activate Cd4 expression (T. Egawa and D.R.L., unpublished data). This failure to activate Cd4 expression in cytotoxic cells is not due to the loss of Cd4 expression potential, because germline deletion of S4 results in robust Cd4 expression in Cd8+ cells, and E4p-Cd4 promoter reporter constructs exhibit strong transcriptional activity upon delivery into mature Cd8+ cells (J.R.H. and D.R.L., unpublished data). Thus, S4 initiates silencing of Cd4 in developing cytotoxic cells but is completely dispensable for the maintenance of that silenced state.

The proximal enhancer initiates an analogous epigenetically active Cd4 expression state in Cd4+ helper cells. Germline deletion of E4p abrogates the upregulation of Cd4 expression at the DN4-to-DP transition during T cell development. MHC class II–specific thymocytes are positively selected in mice homozygous for deletion of E4p (Cd4∆E4PA/E4PA), but these thymocytes are fewer in number in Cd4∆E4PA/E4PA than in wild-type mice, and they display only moderate and unstable CD4 expression. Indeed, in vitro or in vivo proliferation of Cd4∆E4PA/E4PA helper cells results in the gradual loss of CD4 expression. In contrast, Cre-mediated deletion of loxp-flanked E4p in mature helper cells does not affect CD4 expression, even after multiple cell divisions in vitro and in vivo. Thus, E4p is required for the initiation of stable high expression of Cd4 but is also dispensable for its maintenance.

The finding that established silencing of Cd4 can be dissociated from the presence of S4 suggests the existence of a set of genes that epigenetically maintain such silencing independently of S4. As T cells undergo multiple rounds of cell division after activation, these genes would need to both suppress Cd4 re-expression (since Cd8+ cells are able to express Cd4) and actively pass the silenced state from parental cells to daughter cells independently of S4. To identify such putative trans-acting factors, we performed genetic screens with pooled retroviral short hairpin RNA (shRNA) libraries targeting the entire mouse genome. From these screens, we identified DNA methyltransferase 1 (encoded by Dnmt1) as a key factor in Cd4 silencing. Subsequent locus-wide methylation analyses revealed greater methylation of Cd4 in DN, DP and CD8+ cells than in Cd4+ cells. We further found that the DNA-methylation patterns of Cd4 in Cd4+ T cells and CD8+ T cells were dependent on E4p and S4, respectively. E4p-dependent demethylation of the Cd4 locus during the transition from DP thymocyte to Cd4+CD8− thymocyte was achieved in the absence of cell division, consistent with the engagement of an active enzymatic process rather than passive demethylation. Our results provide a description of the epigenetic molecular machinery essential for the heritable regulation of Cd4 expression. Furthermore, they indicate that regulation of Cd4 in mature T lymphocytes provides a unique opportunity with which to delineate the epigenetic mechanisms involved in establishing and maintaining gene expression or heritable silencing.

RESULTS
Unbiased screen for regulators of heritable silencing of Cd4

The factors that mediate true epigenetic silencing of gene expression during T cell development remain poorly characterized. To identify trans-acting factors critical for maintaining silencing of Cd4 in cytotoxic (Cd4−CD8+) T cells, we performed an unbiased, genome-wide retroviral shRNA screen (Supplementary Fig. 1a). First, we obtained Cd8+ cells from the spleen and lymph nodes of mice hemizygous for Cd4 with loxp-flanked S4 and with a tamoxifen-inducible gene encoding Cre knocked into the ubiquitously expressed Ubc locus (Cd4S4−L2−/Ubc-Cre-ER mice), then activated the cells with antibody to the invariant signaling protein CD3 (anti-CD3) and antibody to the costimulatory molecule CD28 (anti-CD28), in the presence of OH-tamoxifen to achieve deletion of S4. We hypothesized that deletion of S4 would allow maximal sensitivity and ensure the identification of only true epigenetic modifiers by eliminating the possibility of S4 activity in mature Cd8+ cells. After 18 h of activation, we transduced cells with pools of a retroviral shRNA library (‘shRNA virus’) and expanded cell populations for 9 d with interleukin 2 (IL-2). On day 5, we enriched the populations for Cd4+ cytotoxic cells through the use of anti-Cd4 magnetic beads. On day 10, a small percentage (~0.5%) of pooled shRNA virus–infected cells expressed Cd4 in addition to Cd8, but there was no expression of Cd4 in mock-infected cells (Fig. 1a). We sorted these Cd4+CD8+ cells on the basis of cell-surface-marker expression, amplified the shRNAs integrated into their genome by PCR, then cloned and sequenced the shRNAs. Notably, 83% of the shRNAs isolated from infected Cd4+CD8+ cells were specific for Dnmt1 (we independently identified two different shRNA clones targeting Dnmt1) (data not shown), which indicated that DNA methylation might be important for maintaining the silencing of Cd4 in fully differentiated cytotoxic T cells.

DNA-methylation machinery maintains Cd4 silencing

To confirm the results of our screen and to determine if DNA-methylation enzymes are important for silencing Cd4, we interfered with DNA-methyltransferase activity by using shRNA-mediated knockdown of Dnmt1 and mice with targeted mutations in genes encoding DNA methyltransferases. Cytotoxic T cells activated by crosslinking of CD3 and CD28 and transduced with the Dnmt1-specific shRNAs identified in the screen exhibited higher surface expression of Cd4 than that of cells transduced with control (non-targeting) shRNA (Supplementary Fig. 1b and data not shown). To rule out the possibility of off-target shRNA effects, we also assessed Cd4 expression after genetic manipulation of DNA-methyltransferase activity. Since deletion of Dnmt1 leads to lymphocyte death after a limited number of cell divisions, we analyzed the maintenance of Cd4 silencing through the use of a hypomorphic mutation in the Dnmt1 locus, in which Dnmt1 expression is reduced to ~10% that of the wild-type locus. In mice with T cells homozygous for the hypomorphic mutation (Dnmt1−/−), demethylation of Cd4 expression was observed in vitro and proliferation (Fig. 1b and data not shown). Further reduction of Dnmt1 expression in these cells through the use of Dnmt1-specific shRNA led to Cd4 expression on ~10% of the cytotoxic cells (Fig. 1b). If this result reflected a requirement for Dnmt1-mediated maintenance methylation in the silencing of Cd4, then Cd4 expression would be expected to increase progressively with successive cell divisions due to passive DNA demethylation. To investigate this, we transduced Cd8+ T cells with control or Dnmt1-specific shRNA, then labeled the cells with the fluorescent dye e670 and quantified Cd4 expression through multiple rounds of cell division, as assessed by dye dilution. Like the division-tracking dye CFSE, e670 binds covalently to cellular proteins during staining and is distributed evenly in daughter cells upon cell division. Consistent with the proposal of a role for Dnmt1-mediated maintenance methylation in the silencing of Cd4, we found increased Cd4 expression with increased numbers of cell divisions (Supplementary Fig. 1c).

To better assess the role of the machinery that maintains DNA methylation in the silencing of Cd4, we monitored Cd4 expression...
in long-term in vivo cell-proliferation assays. First, we adoptively transferred 1 × 10^6 to 1.5 × 10^6 Dnmt1-hypomorphic (Dnmt1^hip/hip^) cytotoxic T cells into lymphopenic hosts deficient in recombination-activating gene 2 (and thus deficient in T cells). After 2–3 weeks, 10–20% of transferred cells expressed CD4 in addition to CD8 (data not shown). Since Dnmt1 deficiency can compromise proliferation, we attempted to diminish the methylation content further in Dnmt1-hypomorphic mice by eliminating the alternative methylation machinery of Dnmt3a and Dnmt3b. The methyltransferases Dnmt3a and Dnmt3b, called ‘de novo methyltransferases’ for their ability to methylate completely unmethylated CpG dinucleotides, have also been linked to the maintenance of DNA methylation 10,11, and their deletion did not substantially affect T cell proliferation (data not shown). On a Dnmt1-hypomorphic background, deletion of Dnmt3b had weaker effect on CD4 expression in cytotoxic T cells than did deletion of Dnmt3a (data not shown). Notably, deleting Dnmt3a in Dnmt1-hypomorphic and Dnmt3b-heterozygous mice resulted in robust CD4 expression (>75% CD4^+) after in vivo population expansion (Fig. 1c). These data demonstrated that the DNA-methylation machinery was critical for ensuring stable silencing of Cd4 in cytotoxic T cells.

**S4-dependent hypermethylation of DNA in the cytotoxic lineage**

The requirement for DNA methyltransferases in the silencing of Cd4 indicated that there would be differences between helper T cells and cytotoxic T cells in the 5’-methyl-cytosine (5Mc) modifications of CpG dinucleotides within the Cd4 locus and that the differences could be dependent on S4. To test this hypothesis, we isolated genomic DNA from peripheral wild-type Cd4^+ helper T cells and CD8^+ cytotoxic T cells, as well as CD4^+CD8^+ cytotoxic T cells homozygous for deletion of S4 in Cd4 (Cd4^+/-/-/-), then enriched the DNA for sequences ~75 kb upstream to ~75 kb downstream of Cd4 by bisulfite CATCH-Seq (‘clone-adapted template-capture-hybridization sequencing’); this method uses bacterial artificial chromosome (BAC) clone templates to generate probes for target-capture hybridization–based enrichment of a locus, followed by bisulfite sequencing 12. CATCH-Seq resulted in >30x sequencing coverage for 97.6% of target CpG motifs per sample, on average, and was highly reproducible for biological replicates (Supplementary Fig. 2). By comparing the variance in methylation levels at each CpG dinucleotide in wild-type CD4^+ and CD8^+ cells and Cd4^+/-/-/- and Cd4^+/-/-/- cells, we identified a strongly ‘differentially methylated region’ (DMR) from ~3.2 kb to ~0.7 kb relative to the Cd4 TSS (called the ‘TSS-proximal DMR’ here) (Fig. 2a); this region included eight of the ten CpG dinucleotides exhibiting the most variance in methylation across the samples and was within a region of ~7 kb (Fig. 2b) that included 26 of the 30 most variably methylated CpG dinucleotides. This DMR was hypermethylated in CD8^+ cells relative to its methylation in CD4^+ cells (22 of 55 CpG motifs exhibited >40% methylation in CD8^+ cells, and more than twice as much methylation in CD8^+ cells as that in CD4^+ cells; Fig. 2b and Supplementary Fig. 3). Notably, this TSS-proximal DMR straddled S4 and overlapped the Cd4 promoter as well as a ‘maturation’ enhancer (adjacent to S4) needed to initiate stable CD4 expression in mature cells of the helper lineage 13 (P.D.I. and D.R.L., unpublished results), which indicated that the DMR might control functional cis-acting elements. We confirmed the existence of the DMR at a subset of CpG motifs by amplicon sequencing and digestion with methylation-sensitive restriction enzymes (Supplementary Fig. 4a–c). Furthermore, the hypermethylation of the Cd4 locus in the cytotoxic lineage was silencer dependent, as methylation patterns in Cd4^+/-/-/- and CD4^+/-/-/- peripheral T cells closely resembled those of wild-type CD4^+ cells (Fig. 2b and Supplementary Fig. 4c).

To ascertain if the hypermethylation pattern observed in CD8^+ T cells was stable, we also assessed DNA methylation after multiple cell divisions. Wild-type CD4^+ and CD8^+ cells and Cd4^+/-/-/- CD4^+CD8^+ cells that had undergone more than five divisions (as assessed by dilution of CFSE) following in vitro stimulation maintained DNA-methylation patterns similar to those of their precursors analyzed immediately ex vivo (Fig. 2c and Supplementary Fig. 4d). Following injection into lymphopenic hosts, these methylation patterns were conserved after 20 d and over ten cell divisions (Supplementary Fig. 4e). Thus, consistent with the proposal of a direct role for DNA methylation in regulating the heritable silencing of Cd4, S4 dictated hypermethylation of the Cd4 locus in cytotoxic T cells, and this hypermethylation was, in turn, stable through multiple cell divisions.

**Hypermethylation of the Cd4 locus in T cell progenitors**

The finding of heritable hypermethylation of Cd4 in CD8^+ T cells and hypomethylation of Cd4 in CD4^+ T cells suggested that the locus undergoes selective de novo methylation as DP cells differentiate toward the cytotoxic lineage. To determine if this was indeed the case, we analyzed DNA methylation by CATCH-Seq, as well as amplicon sequencing. Unexpectedly, we found that the Cd4 locus was already hypermethylated both at the DN3 stage and DP stage (Fig. 3a and Supplementary Fig. 4f,g). Most of the methylated CpG dinucleotides were retained in CD8^+ single-positive (CD8SP); we use the ‘SP’ suffix throughout to denote maturing helper and cytotoxic
**Figure 2** Silencer-dependent DMR in the first intron of Cd4. (a) CpG motifs with >30x coverage, within ~75 kb of the Cd4 TSS (chromosome 6, positions 124749635–124906460 of the UCSC Mus musculus genome assembly mm9), assessed by CATCH-seq analysis of genomic DNA in naive (Thy-1.2+CD4+CD25−CD62L−CD25+) wild-type CD4+ cells, wild-type CD8+ cells and Cd4<sup>S4A/S4A</sup> CD4+CD8+ cells isolated from lymph nodes, presented as variance at each CpG across all samples (vertical axis) versus genomic position (horizontal axis), with genes, S4 and E4P below, 98–98.5% of targeted CpG motifs were captured in each sample at >30x coverage, and median CpG coverage exceeded 300x. (b) Frequency of the methylation of CpG motifs at positions +6200 to –669 relative to the Cd4 TSS (chromosome 6, positions 124832027–124838896 of mm9 (middle of region analyzed in (a)) in wild-type CD4+ cells (WT CD4+), Cd4<sup>S4A/S4A</sup>, Cd4<sup>S4A/S4A</sup> CD4+CD8+ cells (Cd4<sup>S4A/S4A</sup>CD8+) and wild-type CD8+ cells (WT CD8+) (two biological replicates per group); red line underlines CpG dinucleotides in S4 (gaps correspond to deletion of these in Cd4<sup>S4A/S4A</sup> mice); black arrow indicates the Cd4 TSS. (c) Frequency of the methylation of CpG motifs (positions as in b) in CFSE-labeled wild-type CD4+ cells (two biological replicates), Cd4<sup>S4A/S4A</sup>CD4+CD8+ cells (one biological replicate) and wild-type CD8+ cells (two biological replicates) stimulated in vitro with anti-CD3, anti-CD28 and IL-2, followed by locus enrichment and high-throughput bisulfite sequencing (as in a) for those cells that had undergone at least six divisions after 5 d. Data are from two independent experiments.

**DMR methylation does not grossly alter chromatin structure**

We next considered how hypermethylation of the Cd4 locus might affect silencing. As DNA methylation can regulate nucleosome stability and positioning in some contexts<sup>14,15</sup>, we evaluated the possibility that methylation of the Cd4 locus might affect nucleosomes and, hence, chromatin compaction. Thus, we used micrococcal nuclease digestion and CATCH-seq to identify nucleosomes from diverse samples, including DN3 cells, DP cells, CD4+ cells, CD8+ cells, Cd4<sup>E4P/E4P</sup> DP cells, Cd4<sup>E4P/E4P</sup> CD4+ cells, Cd4<sup>S4A/S4A</sup> DP cells and Cd4<sup>S4A/S4A</sup> CD8+ cells. We found that the loss of nucleosomes at position ~+3.5 kb (relative to the TSS), immediately downstream of the TSS-proximal DMR, correlated strongly with Cd4 expression (<sup>Supplementary Fig. 5</sup>). However, DNA-methylation content did not correlate well with differences between samples in nucleosome positioning, which indicated that this was probably not a critical function of 5mC marks in the Cd4 locus in facilitating silencing.

**Hypomethylation of Cd4 correlates with stable Cd4 expression**

The hypomethylation of Cd4 in helper lineage cells led us to hypothesize that the removal of 5mC marks is critical for heritable, high Cd4 expression. To test this, we assessed whether the amount of 5mC in Cd4 was dependent on the proximal enhancer. Cd4<sup>E4P/E4P</sup> CD4+ helper T cells exhibit unstable Cd4 expression upon cell division<sup>2</sup>. Notably, in naive Cd4<sup>E4P/E4P</sup> CD4+ cells there was hypomethylation of the Cd4 TSS-proximal DMR, with 5mC content approaching.

**Figure 3** Hypomethylation of Cd4 in immature thymocytes. (a) Frequency of the methylation of CpG dinucleotides (positions top) as in Fig. 2b), assessed by CATCH-seq analysis of genomic DNA from sorted populations of wild-type thymocytes at the following stages: DN3 (Thy-1.2+Lin−CD25−CD44−) (one biological replicate), DP (TCRβ<sup>+</sup>CD24<sup>+</sup>CD69−CD4−CD8+) (two biological replicates), CD4SP (TCRβ<sup>+</sup>CD24<sup>+</sup>CD69<sup>+</sup>CD4−CD8<sup>-</sup>) (two biological replicates) and CD8SP (TCRβ<sup>+</sup>CD24<sup>-</sup>CD69<sup>-</sup>CD4−CD8<sup>+</sup>) (two biological replicates). (b) Frequency of the methylation of CpG motifs (as in a), assessed by CATCH-seq analysis of DP cells sorted from Cd4<sup>S4A/S4A</sup> mice (two biological replicates). Data are from two independent experiments.
that observed in mature wild-type CD8+ cells (Fig. 4a) and correlating with unstable CD4 expression. Further, when we stimulated naive Cd4E4P/E4Pa CD4+ cells to proliferate in vitro and analyzed Cd4-methylation patterns locus wide in CD4+ cells and CD4- cells after over five cell divisions, those cells that lost CD4 expression exhibited more demethylation of the TSS-proximal DMR (Fig. 4b). Thus, in helper T cells, hypomethylation of the Cd4 locus correlated with more stable maintenance of CD4 expression (continued CD4 expression after more than five cell divisions), while hypermethylation correlated with loss of CD4 expression. In Cd4E4P/E4Pa thymic DP cells, which lack CD4 expression, the Cd4-methylation pattern was most similar to that in wild-type DN3 cells, with hypermethylation ±1 kb from the location of E4P (Supplementary Fig. 6). Together these results suggested that E4P contributed to stable CD4 expression in the helper lineage by facilitating heritable demethylation of the Cd4 locus.

**Diminished DNMT1 ‘rescues’ CD4 expression**

To determine if there might be a causal link between hypomethylation of the Cd4 locus and heritable CD4 expression, we interfered with DNMT1 expression in E4P-deficient helper cells. We sorted naive CD4+ cells from mice with loxP-flanked Dnmt1 alleles with or without expression of Cd4-Cre (Dnmt1L2/L2Cd4-Cre+ or Dnmt1L2/L2Cd4-Cre-), and from mice with T cells hemizygous for the Dnmt1chip hypomorphic mutation (Dnmt1L2/L2chipCd4-Cre+), all on a Cd4E4Pa/E4Pa background, and analyzed CD4 expression at various time points after activation. We measured expression of CD4 protein, as it correlates with expression of Cd4 mRNA in Cd4E4Pa/E4Pa T cells. At 72 h, while 76.2% of Cd4E4Pa/E4PaDnmt1L2/L2Cd4-Cre+ (Dnmt1-sufficient) cells maintained CD4 expression, 94.9% and 87.6% of Cd4E4Pa/E4PaDnmt1L2/L2Cd4-Cre+ and Cd4E4Pa/E4PaDnmt1L2/L2chipCd4-Cre+ cells, respectively, expressed CD4 (Fig. 5a). Cd4+ E4Pa/E4PAdnmt1L2/L2chipCd4-Cre- cells exhibited a mean fluorescence intensity of CD4 ~30% higher than that of Cd4E4Pa/E4PaDnmt1L2/L2Cd4-Cre+ cells at 72 h (Fig. 5b), which indicated higher CD4 expression in the cells that remained CD4+. At 96 h and 120 h, we also observed larger proportions of CD4+ cells (20–25% higher) and higher mean fluorescence intensity of CD4 (40–47% higher) in Cd4E4Pa/E4PaDnmt1L2/L2chipCd4-Cre- populations than in Dnmt1-sufficient control populations (Supplementary Fig. 7a,b). Notably, these results were not due to differences in proliferative capacity, because the fraction of CD4+ cells was...
our observation that DNA demethylation in the helper lineage occurred after Zbtb7b was maximally induced suggested that its product, ThPOK, might have a role in the removal of these methylation marks. To test this hypothesis, we first assessed DNA methylation by ampiclon sequencing of MHC class II–selected, Zbtb7b

Figure 6 Demethylation of the Cd4 locus occurs late in helper-lineage differentiation and is largely independent of ThPOK. (a) Amplicon of Cd4 intron 1 (chromosome 6; Chr 6); positions (blue bar) noted below analyzed in b–d. Filled symbols (above) indicate approximate locations of CpG dinucleotides (not drawn to scale). (b–d) Bisulfite sequencing of the amplicon in a in GFP– thymocytes, GFPmid or GFP– CD4+CD8lo (CD69+HAS+TCRβ+) thymocytes and GFP– CD4SP (CD69–HAS–TCRβ+) thymocytes from Zbtb7b

Demethylation of the DMR during commitment to the helper lineage

We next sought to determine when DNA demethylation occurred in cells of the helper lineage. We examined methylation of the Cd4 TSS-proximal DMR by ampiclon bisulfite sequencing at various stages during positive selection and during differentiation into the helper lineage through the use of a Zbtb7b

ThPOK is partially dispensable for demethylation of Cd4

Our observation that DNA demethylation in the helper lineage occurred after Zbtb7b was maximally induced suggested that its product, ThPOK, might have a role in the removal of these methylation marks. To test this hypothesis, we first assessed DNA methylation by ampiclon sequencing of MHC class II–selected, Zbtb7b

Cell division–independent demethylation of the Cd4 locus

The removal of DNA-methylation marks in the Cd4 locus late in lineage commitment raised the question of whether demethylation is passive,
by way of cell division, or active, through biochemical removal of 5mC marks. Among the 22 CpG motifs of the TSS-proximal DMR that were differentially methylated in cells of the helper lineage relative to that in cells of the cytotoxic lineage, the median ratio of CpG-methylation in DP cells to that in CD4SP cells was 4.7 (mean, 26.8; maximum, 343). Thus, passive demethylation would require an average of at least two cell divisions during differentiation into the helper lineage and as many as nine divisions to explain the demethylation observed at some residues. Published reports have suggested that there is no cell division between the DP stage and CD4SP stage of development\textsuperscript{19,20}, or that a minority (<20%) of CD4SP cells may divide after downregulation of heat-stable antigen and within stages in MHC class II–selected CD4\textsuperscript{+}CD8\textsuperscript{lo} cells. We sorted CD69\textsuperscript{−} DP cells from CD45.2\textsuperscript{+} cells and CD45.1\textsuperscript{+}CD4\textsuperscript{+}CD8\textsuperscript{lo} cells (CD69\textsuperscript{+}HSA\textsuperscript{−}CD4\textsuperscript{+}CD8\textsuperscript{lo}GFP\textsuperscript{+}) incubated in the presence of UDP-glucose, with (+JGT) or without (-JGT) β-glucosyltransferase (key), followed by digestion with MspI (at position 124838036 of chromosome 6; +191 bp relative to the CD4 TSS) and quantitative PCR; results are presented as DNA not digested by MspI, normalized to results obtained for an adjacent amplicon without an MspI site.

At 4 d after injection, we isolated the thymus of each recipient mouse and assessed CFSE intensity and lineage differentiation by flow cytometry (Supplementary Fig. 8d–f). Among both CD45.1\textsuperscript{+} and CD45.2\textsuperscript{+} injected cell populations, we were able to identify pre-selected cells (CD69\textsuperscript{−} DP), positively selected cells (CD4\textsuperscript{+}CD8\textsuperscript{lo}CD69\textsuperscript{+}TCR\textsuperscript{bmed–hi} cells) and mature CD4SP cells (CD4\textsuperscript{+}CD8\textsuperscript{−}CD69\textsuperscript{−}TCRB\textsuperscript{bhi}). All three groups had similarly high CFSE labeling, which indicated no difference in cell division after injection. Notably, the lack of cell division was not due to loss of viability, as these cells were able to differentiate and to modulate their expression of CD69, TCRB, CD4 and CD8. Thus, without evidence of substantial proliferation following positive selection, we concluded that methylation was removed from the Cd4 locus during commitment to the helper lineage via an active biochemical process, independently of cell division.

Oxidation of 5mC to 5-hydroxymethyl-cytosine (5hmC), mediated by the TET (‘ten eleven translocation’) enzymes, has been shown to contribute to active DNA-demethylation pathways\textsuperscript{22}. To determine if TET-mediated oxidation of 5mC in the Cd4 locus might contribute to locus demethylation, we assessed the amount of the 5hmC modification by T4-phage β-glucosyltransferase (T4-JGT)-mediated restriction-enzyme protection, followed by quantitative PCR. We isolated genomic DNA from DP cells, CD4\textsuperscript{+} cells and MHC class II–selected CD4\textsuperscript{+}CD8\textsuperscript{lo} cells (CD69\textsuperscript{−}HSA\textsuperscript{−}CD4\textsuperscript{+}CD8\textsuperscript{lo}GFP\textsuperscript{−} cells from Zbtb7b\textsuperscript{GFP/+} mice) and incubated the DNA with or without T4-JGT in the presence of UDP-glucose, before digestion of the DNA with MspI, a restriction enzyme sensitive to modified 5hmC. T4-JGT transfers UDP-glucose specifically to 5hmC residues, which blocks the digestion of CCCG motifs by MspI. In DP cells and CD4\textsuperscript{+} cells, we found very little protection (<20%) by T4-JGT of a CCCG

![Figure 7](https://example.com/figure7.jpg)
motif at +190 bp relative to the Cd4 TSS, which was differentially methylated in cells of the helper lineage relative to its methylation in cells of the cytotoxic lineage (Fig. 7a). However, we found significantly greater T4-βGT-mediated protection of the same motif in MHC class II–selected CD4+CD8lo cells (~40%) (Fig. 7a), consistent with TET-dependent 5hmC-mediated demethylation in differentiating T cells of the helper lineage. Notably, this CpG lies near the middle of the TSS-proximal DMR, and its methylation status during T cell differentiation is representative of the other dynamically and differentially methylated CpG dinucleotides in the TSS-proximal DMR.

To confirm and expand on those results, we performed oxidative-bisulfite amplicon sequencing of a group of three representative CpG motifs located at positions +1407 to +1487 relative to the TSS. While both 5mC and 5hmC are ‘read’ as cytosine residues after bisulfite treatment, 5hmC can be oxidized by KRuO4 to 5-formylcytosine, which is then ‘read’ as thymine after bisulfite treatment23. Thus, bisulfite treatment, with or without oxidation, can be used to distinguish 5mC modifications from 5hmC modifications. In DP cells, we found that all three CpG motifs were highly methylated (>90% 5mC or 5hmC; bisulfite treatment alone), with small amounts of 5hmC at two CpG motifs (<20% 5hmC at CpG 1 and CpG 2) and moderate amounts at a third CpG motif (<50% 5hmC at CpG 3) (Fig. 7b). While the overall methylation content remained similar, we observed less 5mC and more 5hmC in post-selection CD4+CD8hi cells than in DP cells (Fig. 7b,c), in keeping with our results reported above (Figs. 6b and 7a and Supplementary Fig. 4g). We note that CD4+CD8hi cells are predominantly MHC class II selected (a ratio of >2:1, MHC class II selected to MHC class I selected)16. Together these results were consistent with our finding that demethylation of the TSS-proximal DMR after selection on MHC class II did not require cell division and suggested that it might be achieved enzymatically via a hydroxymethylated intermediate.

**DISCUSSION**

The molecular mechanism for heritable epigenetic silencing of Cd4 in cytotoxic T cells has remained elusive despite characterization of the key cis- and trans-acting factors required for establishment of the silenced state. We found here that DNA methyltransferases were needed to maintain silencing in cytotoxic-lineage T cells, and we linked this requirement to methylation of the Cd4 locus. Conversely, our data indicated that stable Cd4 expression in helper-lineage T cells was regulated by proximal enhancer–dependent demethylation of the Cd4 locus. Unexpectedly, the Cd4 TSS-proximal DMR was methylated early in development, in both DN thymocytes and DP thymocytes, which indicated that a critical function of the silencer was to antagonize demethylation in the cytotoxic lineage. Finally, demethylation of the DMR in helper lineage thymocytes seemed to involve an active enzymatic process, probably mediated by TET-dependent oxidation of methylated cytosine residues. Thus, our results suggest that heritable silencing of Cd4 versus expression of Cd4 is directed by a DNA-demethylation switch under the control of the silencer and proximal enhancer.

Our results indicate that methylation of the Cd4 locus is necessary, but not sufficient, for stable silencing of Cd4. Indeed, DP cells expressed amounts of Cd4 similar to those expressed by helper T cells, but exhibited hypermethylation of the Cd4 locus comparable to that of cytotoxic T cells. Such uncoupling of methylation of the TSS-proximal DMR from transcription indicates that methylation and demethylation of the Cd4 locus is critical for establishing heritable Cd4 expression states rather than transcriptional activity. Still, the following question remains: how do DP cells express Cd4 in the face of TSS-proximal methylation? This might be due to developmental stage–specific expression of trans–acting factors. For example, expression of the transcription factor RUNX1 is downregulated at the DN4-to-DP transition2,24, which might contribute to diminished S4 activity and allow Cd4 expression despite hypermethylation of Cd4. Alternatively, or in conjunction with low levels of RUNX, DP cells may express as-yet-unidentified stage–specific activators of Cd4. Further, we note that a handful of CpG dinucleotides in the Cd4 locus were hypomethylated in DP cells and CD4+ cells relative to their methylation in CD8+ and DN cells; hypomethylation of these residues in DP cells might contribute to Cd4 expression in DP cells, while their de novo methylation in CD8+ cells might contribute to silencing of Cd4. Finally, it is possible that cells of the CD8+ lineage express lineage–specific factors, possibly including RUNX3, that act together with 5mC modifications to impose a silenced state.

Our data suggest that stable expression of Cd4 in the helper lineage depends on an E4P-directed active DNA-demethylation process in MHC class II–selected thymocytes: first, E4P was required for hypomethylation of the Cd4 locus in the Cd4+ lineage; second, there was little to no cell division between the DP (hpermethylated) stage and CD4SP (hypomethylated) stage of development; third, we found 5hmC in MHC class II–selected CD4+CD8lo cells at CpG dinucleotides in the TSS-proximal DMR, which were demethylated during the DP-to-CD4SP transition, consistent with oxidation of 5mC by TET enzymes. E4P becomes dispensable in mature cells25, in which a ‘maturation enhancer’, putatively located adjacent to the silencer element in Cd4 intron 1 (ref. 13, and P.D.I. and D.R.L., unpublished data), and within the TSS-proximal DMR, programs Cd4 expression. Thus, it seems likely that the critical function of E4P is to effect demethylation of TSS-proximal sites across the promoter and sequences flanking intronic regulatory elements, which then allows the maturation enhancer to direct Cd4 expression. We note, however, that TET enzymes can further oxidize 5hmC to 5-formylcytosine and 5-carboxycytosine25–27, which are ‘read’ as ‘unmodified’ cytosine residues by bisulfite sequencing23. Thus, we cannot rule out the possibility that the unmethylated CpG motifs identified in differentiating cells of the helper lineage represent 5-formylcytosine or 5-carboxycytosine. Further studies are needed to determine how demethylation and/or oxidation of cytosine residues is (are) critical for establishing heritable Cd4 expression.

Central questions raised by our study are how E4P directs demethylation in the helper lineage and how S4 maintains the minimally altered methylation pattern following the transition from the DP stage to the CD8+ lineage. While it is possible that TET enzymes are recruited to E4P upon signaling via the TCR, we also note that the TSS-proximal DMR contained some 5hmC modifications in wild-type DP cells and that E4P was responsible for hypomethylation of E4P-proximal CpG motifs in DP cells. Thus, we are tempted to hypothesize that E4P recruits TET enzymes in DP cells or in cells at earlier stages, to poise the Cd4 locus for demethylation before positive selection and lineage commitment. Could S4 then inhibit the recruitment or activity of TET enzymes in the Cd4 locus in MHC class I–selected cells? RUNX3 expression is induced in MHC class I–selected cells during the DP-to-CD8SP transition28, and the RUNX fusion proteins found in acute myeloid leukemia have been shown to recruit DNA methyltransferases to target genes, presumably through an indirect mechanism29,30. Thus, it is possible that RUNX3 might recruit DNA methyltransferases to the Cd4 locus to ensure maintenance of methylation. Future studies should determine if and how TET enzymes are targeted to the Cd4 locus in an E4P–dependent manner and whether DNA
methyltransferases are recruited in an S4- and RUNX3-dependent manner during the CD4+CD8lo- to-CD8SP transition.

**CD4** is arguably the best-characterized locus in vertebrates for the study of heritability, but the mechanisms by which its heritable states are controlled have remained elusive for years. Our findings suggesting that the DNA-methylation machinery is critical for the establishment and maintenance of silencing and that demethylation is critical for heritable expression represent an advance in this field and offer new opportunities for delineation of the signaling pathways involved in thymocyte lineage ‘choice’. Our results have also established **CD4** as a unique model with which to elucidate how DNA demethylation is effected and regulated. Further investigation of how the **CD4** locus is controlled via DNA methylation might thus provide important insights into how fully differentiated somatic cells achieve heritable states of gene expression.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** NCBI BioProject: aligned bisulfite CATCH-Seq data and nucleosome CATCH-Seq data, [PRJNA282735](https://www.ncbi.nlm.nih.gov/PROJECT/PRJNA282735). NCBI SRA Analysis: percent cytosine methylation from bisulfite CATCH-Seq analysis, [SRZ100453](https://www.ncbi.nlm.nih.gov/SRA/SRZ100453).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

M.S. performed E4P rescue experiments, proliferation assays in thymus and T4-JBT analysis; J.R.H. did the genetic screen and follow-up analyses; M.S. and K.D. did the analyses of Cd4 locus-wide methylation and nucleosome sequencing, with bioinformatics support from D.A.; P.D.I. performed oxidative bisulfite analysis; M.S. and C.G. performed amplicon bisulfite sequencing; S.G. and M.R.G. provided the mouse shRNA retroviral pools; and M.S., J.R.H. and D.R.L. designed the experiments and wrote the manuscript with input from the other authors.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. Dnmt1L-2 mice35 and Dnmt1chip mice37 were a gift from R. Jaenisch. Dnmt3L-2 mice35 and Dnmt3H-2 mice35 were from the Mutant Mouse Regional Resource Center. Cd46b-2 mice, Cd46a-2 mice, and Zbtb7b(−/−) mice35 have been described. Wild-type C57BL/6 mice, Cd4-Cre mice, Ubc-CreERT2 mice, H2-AK-/- mice35, B2m−/− mice36, Rag2-genet mice, LysM−/− mice, Ly5.1−/− mice, Ly5.2−/− mice and Tg(TcarTerb)425Chn (OT-III) mice were from the Jackson Laboratory. All mice were maintained under specific pathogen-free conditions in the Skirball Institute Animal Facility. All experiments were performed in accordance with the protocol approved by the IACUC at the NYU School of Medicine.

shRNA screen. The mouse shRNA mini library37 was used to generate ten retroviral pools, each comprising ~6,000 shRNA clones38, CD62L−/CD25+CD8+ cells, isolated from the spleen and lymph nodes of Cd46b-1/2 Ubc-CreERT2 mice, were cultured for 16 h with anti-CD3 (0.25 µg/ml; identified below) and anti-CD28 (1 µg/ml; identified below) in the presence of OH-tomoxifen (400 nM) before transduction with the retroviral pools. After 7 d of culture in the presence of IL-2 (100 U/ml), populations underwent enrichment for CD4+ cells through the use of a CD4+ MACS column. After 3 d, CD4+CD8+ DP cells were sorted and their genomic DNA was isolated. For the identification of candidate shRNA, the shRNA region of the transduced virus was amplified by PCR, cloned, and sequenced. Individual shRNAs were from the Open Biosystems library or were synthesized. Two shRNA cloning Dnmt1+ were identified (5′-GTACATTTTTACATGTTTGGAAA-3′ and 5′-TCCCGAATGATCCACAAA-3′).

Flow cytometry and sorting. Monoclonal antibodies were as follows: anti-CD69 (H1.2F3) and anti-IFN-γ (XMG1.2) (both from BD Biosciences); anti-CD4 (RM4-5), anti-CD8α (53-6.7), anti-TCR (H57-597), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-CD45.1 (A20), anti-CD45.2 (104), CD25 (PC61) (all from eBioscience). CFSE (carboxyfluorescein diacetate succinimidyl ester) and e670 were from Molecular Probes. After staining with antibodies and DAPI (4,6-diamidino-2-phenylindole; diacetate succinimidyl ester) and e670 were from Molecular Probes. After staining with antibodies and DAPI (4,6-diamidino-2-phenylindole; Molecular Probes), cells were analyzed with an LSRII flow cytometer (BD Biosciences). Post-sort sample purity was >98%. In some cases, anti-CD4, anti-phyceroerythrin and anti-B220 magnetic beads (Miltenyi) were used for enrichment and depletion on the AutoMACS platform (Miltenyi) before sorting. Flow cytometry data were analyzed with FlowJo software (TreeStar).

Cell culture. Tissue culture plates were incubated overnight with polyclonal goat antibody to hamster IgG (0859898; MP Biomedicals), then washed three times with PBS, then purified CD4+CD8−CD25+CD62L+ naive CD4+CD8+CD25−CD62L−CD44+T cells were added, along with anti-CD3 (0.25 µg/ml; 145-2C11; eBioscience) and anti-CD28 (1 µg/ml; 37.5.1; eBioscience). At day 3, cultures were supplemented with 100 U/ml recombinant IL-2 (G4-102; BioXcell) and 100 U/ml recombinant IL-1β (PeproTech). For knockdown of Dnmt1, the Dnmt1-specific shRNA sequences were inserted into a miR-30-based hairpin of the vector pMSCV-LMP according to the manufacturer’s instruction (Open Biosystems). Retroviruses were packaged in Plat-E cells39 by transient transfection with TransIT 293 (Mirus Bio). Cells were transduced by ‘spin infection’ at 1,200 g at 30 °C for 90 min in the presence of 10 µg/ml polybrene (Sigma).

Methylation analysis. Genomic DNA was isolated with Purelink genomic DNA isolation kits (Invitrogen). For locus-wide bisulfite sequencing, CATCH-seq was performed as described35 with Bac clone RP24330J12 (BACPAC Resource Center, Children’s Hospital Oakland Research Institute). For amplicon sequencing, bisulfite conversion was performed with an EpiTect Bisulfite Kit (Qiagen). Methylation analysis. Methylation analysis. Nucleosome analysis. Nucleosomes were prepared as described42. 1.1 × 10⁶ cells were lysed in digestion buffer (50 mM Tris-HCl, pH 7.5, 1 mM CaCl₂ and 0.2% Triton X-100) supplemented with inhibitors of proteases and deacetylases. Nuclei were then treated for 5 min at 37 °C with 12 U micrococcal nuclease (Worthington Biochemical) in 135 µl digestion buffer to produce >90% mono-nucleosomes. The reaction was quenched by the addition of EDTA to a concentration of 25 mM EDTA and EGTA to a concentration of 10 mM. Samples were spun for 5 min at 2,500 g and supernatants with solubilized mononucleosomes were reserved (digestion supernatants). Pelleted nuclei were then lysed twice for 1 h on ice in lysin buffer (150 µl of 1 mM Tris-HCl, pH 7.5, and 0.25 mM EDTA; supplemented with inhibitors of proteases and deacetylases), following gentle sonication. After removal of nuclear debris by centrifugation (5 min at 11,000 g), nuclear lysis supernatants were pooled with digestion supernatants. Mononucleosome fragments were then subjected to CATCH-seq analysis.

Statistical analysis. Student’s t-test was used for statistical analysis, and P values of <0.05 were considered significant. All technically valid and high-quality (e.g., high coverage for sequencing) data were included for analysis. While a few biological replicates with lower high-throughput sequencing coverage were not included, they did show similar trends. No randomization or blinding was used.

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