HHS Public Access

Author manuscript

Nat Immunol. Author manuscript; available in PMC 2016 January 01.

Published in final edited form as:

Nat Immunol. 2015 July ; 16(7): 746–754. doi:10.1038/ni.3198.

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

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Abstract

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. *Cd4* regulation in helper and cytotoxic T cells exemplifies this process, with enhancer- and silencer-regulated establishment of epigenetic memories for stable gene expression and repression, respectively. Using a genetic screen, we identified the DNA methylation machinery as essential for maintaining *Cd4* silencing in the cytotoxic lineage. Further, we found a requirement for the proximal enhancer in mediating removal of *Cd4* DNA methylation marks, allowing for stable expression in T helper cells. These 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.

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ACCESSION CODES

BioProject ID PRJNA282735

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

J.R.H. did the genetic screen and follow-up analyses. M.S. and K.D. did the *Cd4* locus-wide methylation and MNase analyses with bioinformatics support from D.A. M.S. and C.G. performed amplicon bisulfite sequencing. M.S. performed E4p rescue experiments, proliferation assays in thymus and T4-βGT analysis. P.D.I. performed oxidative bisulfite analysis. S.G. and M.R.G. provided the murine shRNA retroviral pools. J.R.H., M.S., and D.R.L. designed the experiments and wrote the manuscript with input from the other authors.

INTRODUCTION

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, for example receptor gene segment rearrangements in B and T cells, the genome sequence remains unchanged as cells adopt new identities. Stable lineage commitment requires establishment of heritable patterns of gene expression or repression without alteration of DNA sequences, via epigenetic modifications. Despite a rapidly growing body of work that describes putative epigenetic regulation, physiological models in which epigenetic modulation can be functionally dissected 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[1]. $CD4^+$ helper and $CD8^+$ cytotoxic T cells develop from common progenitors, based on the specificity of their T cell antigen receptors (TCRs) for peptide-major histocompatibility complex (MHC) class II or class I molecules, respectively. The CD4 and CD8 co-receptors are critical to the development and function of these lineages, as they facilitate TCR binding to MHCII (CD4) and MHCI (CD8). CD4 and CD8 expression defines distinct stages of thymocyte development, during which ordered *Tcr* gene rearrangements occur and serve as developmental checkpoints. Early $CD4^-CD8^-$ double-negative (DN) progenitors transition through four distinct stages before up-regulating CD4 and CD8 to enter the $CD4^+CD8^+$ double-positive (DP) stage of development. DP cells then test their randomly rearranged TCRs for MHCI and II specificity. MHCI-specific cells stably down regulate CD4 to enter into the cytotoxic lineage, while MHCII-specific cells lose CD8 expression and maintain CD4 expression during helper lineage differentiation.

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[1]. Elements required for this regulation have been identified in a series of *in vivo* genetic studies and *in vitro* T cell culture assays[2–6]. These include a 434 bp *cis*-acting silencer (S4), located in the first intron of the *Cd4* locus, and a 430 bp *cis*-acting proximal enhancer ($E4_P$), located 13 kb upstream of the transcriptional start site (TSS). S4 is essential for *Cd4* repression at two different stages of T cell development. First, germline S4 deletion leads to ectopic CD4 expression in DN cells, indicating that it is required for reversible silencing before the DP stage of development. Second, S4 is required for silencing *Cd4* in mature $CD8^+$ cytotoxic cells, since germline S4 deletion results in ectopic CD4 expression in cytotoxic lineage cells. However, Cre-mediated conditional S4 deletion in mature $CD8^+$ lineage cells following their thymic egress does not affect CD4 expression even after multiple cell divisions[5]. Similarly, in mature cytotoxic cells, Cre-mediated deletion of genes encoding members of the RUNX protein complex that binds S4 to initiate *Cd4* silencing[3] fails to activate *Cd4* gene expression (Egawa and Littman, unpublished). This failure to activate *Cd4* expression within cytotoxic cells is not due to the loss of *Cd4* gene expression potential because germline S4 deletion results in robust CD4 expression in $CD8^+$ cells and $E4_P$-*Cd4* promoter reporter constructs exhibit strong activity upon delivery into mature $CD8^+$ cells (J.R.H, D.R.L, unpublished).

Thus, S4 initiates *Cd4* silencing 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[7]. Germline $E4_P$ deletion abrogated CD4 upregulation at the DN4 to DP transition during T cell development. However, a reduced number of MHCII-specific thymocytes were positively selected in $Cd4^{E4P\Delta/E4P\Delta}$ mice, and these cells displayed moderate, but unstable, CD4 expression. Thus, *in vitro* or *in vivo* proliferation of $Cd4^{E4P\Delta/E4P\Delta}$ helper cells resulted in the gradual loss of CD4. In contrast, Cre-mediated deletion of a *lox*P-flanked $E4_P$ in mature helper cells did not affect CD4 expression, even after multiple cell divisions *in vitro* and *in vivo*. Thus, $E4_P$ is required for the initiation of stable high expression of CD4, but is also dispensable for its maintenance.

The finding that established *Cd4* silencing can be disassociated from the presence of S4 suggests the existence of a set of genes that epigenetically maintain 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 possess the capacity to express CD4) and actively pass the silenced state from parental to daughter cells independently of S4. To identify these putative *trans*–acting factors, we performed genetic screens using pooled retroviral shRNA libraries targeting the entire mouse genome. From these screens, DNA methyltransferase I (*Dnmt1*) was identified as a key factor in *Cd4* silencing. Subsequent locus-wide methylation analyses revealed *Cd4* hyper-methylation in DN, DP and $CD8^+$ cells compared to $CD4^+$ cells. We further found that in $CD4^+$ and $CD8^+$ T cells the *Cd4* DNA methylation patterns are dependent on $E4_P$ and S4, respectively. $E4_P$-dependent demethylation of the locus during the DP to $CD4^+CD8^-$ thymocyte transition was achieved in the absence of cell division, consistent with the engagement of an active enzymatic process rather than passive demethylation. These results provide the first description of the epigenetic molecular machinery essential for the heritable regulation of *Cd4* expression. Furthermore, they indicate that *Cd4* gene regulation in mature T lymphocytes provides a unique opportunity to dissect the epigenetic mechanisms involved in establishing and maintaining gene expression or heritable silencing.

RESULTS

Unbiased screen for regulators of heritable *Cd4* silencing

The factors that mediate *bona fide* epigenetic silencing of gene expression during T cell development remain poorly characterized. To identify *trans*-acting factors critical for maintaining *Cd4* silencing in cytotoxic T cells ($CD4^-CD8^+$), we performed an unbiased, genome-wide retroviral shRNA screen (Supplementary Fig. 1a). We first activated spleen and lymph node $CD8^+$ cells from $Cd4^{S4\text{-}L/+}$*Ubc*-Cre-ER mice with anti-CD3 and anti-CD28 in the presence of OH-tamoxifen, to delete S4. We hypothesized that S4 deletion would allow for 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, cells were transduced with pools of a retroviral shRNA library and expanded for 9 d with interleukin 2 (IL-2). On day 5, $CD4^+$ cytotoxic cells were enriched using 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 in mock-infected cells (Fig. 1a). These $CD4^+CD8^+$ cells were sorted based on cell surface marker expression, and the shRNAs integrated into their genome were PCR-amplified, cloned and sequenced. Interestingly, 83% of the shRNAs isolated from infected $CD4^+CD8^+$ cells were specific for *Dnmt1* (two different shRNA clones targeting *Dnmt1* were independently identified), indicating that DNA methylation may be important for the maintenance of *Cd4* silencing in fully differentiated cytotoxic T cells.

DNA methylation machinery maintains *Cd4* silencing

To validate the results of our screen and to determine if DNA methylation enzymes are important for *Cd4* silencing, we interfered with DNA methyltransferase activity by using shRNA knockdown and mice with targeted mutations in *Dnmt* genes. Cytotoxic T cells activated by CD3 and CD28 crosslinking and transduced with the *Dnmt1* shRNAs identified in the screen exhibited increased surface CD4 expression compared to cells transduced with a control vector (Supplementary Fig. 1b and data not shown). To rule out off-target shRNA effects, we also tested CD4 expression after genetic manipulation of DNA methyltransferase activity. Since *Dnmt1* deletion leads to lymphocyte death after a limited number of cell divisions[8], we analyzed the maintenance of *Cd4* silencing using a hypomorphic mutation in the *Dnmt1* locus[9], $Dnmt1^{Chip}$, in which Dnmt1 expression is reduced to ~10% of wild-type. In mice homozygous for the hypomorphic mutation ($Dnmt1^{Chip/Chip}$) or hemizygous for the mutation ($Dnmt1^{L/Chip}$*Cd4*-Cre), 1–2 % of cytotoxic T cells upregulated CD4 expression following *in vitro* activation and expansion (Fig. 1b and data not shown). Further reduction of Dnmt1 expression in these cells using *Dnmt1* shRNA led to CD4 expression on ~10% of the cytotoxic cells (Fig. 1b). If this result reflects a requirement for Dnmt1-directed maintenance methylation in *Cd4* silencing, then CD4 expression may be expected to increase progressively with successive cell divisions due to passive DNA demethylation. To examine this, we transduced $CD8^+$ T cells with control or *Dnmt1* shRNAs, then labeled the cells with the fluorescent dye e670 and quantified CD4 expression through multiple rounds of cell division, as assessed by dye-dilution. Similarly to CFSE, e670 binds covalently to cellular proteins during staining and is distributed evenly between daughter cells upon cell division. Consistent with a role for Dnmt1-mediated maintenance methylation in *Cd4* silencing, we found increased CD4 expression with increased numbers of cell divisions (Supplementary Fig. 1c).

To better assess the role of the DNA maintenance methylation machinery in *Cd4* silencing, we monitored CD4 expression in long-term, *in viv*o cell proliferation assays. First, we adoptively transferred *Dnmt1* hypomorphic cytotoxic T cells into T cell-deficient lymphopenic hosts. 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 reduce methylation content further in *Dnmt1* hypomorphic mice by removing alternative methylation machinery, namely Dnmt3a and Dnmt3b. The *de novo* methyltransferases Dnmt3a and Dnmt3b have also been implicated in DNA maintenance methylation[10,11] and their deletion does not significantly affect T cell proliferation (not shown). Compared to *Dnmt3a* deletion on a *Dnmt1* hypomorphic background, *Dnmt3b* deletion had a relatively weak effect on *Cd4* gene expression in cytotoxic T cells (data not

shown). Intriguingly, eliminating Dnmt3a in *Dnmt1* hypomorphic mice resulted in robust CD4 expression (>75%) after *in vivo* expansion (Fig. 1c). These data clearly demonstrate that DNA methylation machinery is critical for ensuring stable *Cd4* silencing in cytotoxic T cells.

S4-dependent, cytotoxic lineage DNA hyper-methylation

The requirement for DNA methyltransferases in *Cd4* silencing implied that there would be differences in 5′-methyl-cytosine (5mC) modifications of CpG dinucleotides within the locus in helper and cytotoxic T cells, and that the differences could be dependent on S4. To test this hypothesis, we isolated genomic DNA from peripheral wild-type $CD4^+$ helper and $CD8^+$ cytotoxic, and $Cd4^{S4\Delta/S4\Delta}$ $CD4^+CD8^+$ cytotoxic T cells, and enriched for the *Cd4* locus flanked on each side by ~75 kb, by CATCH-Seq; this method uses BAC clone templates to generate probes for target capture hybridization-based locus enrichment, followed by bisulfite sequencing[12]. CATCH-Seq resulted in >30x sequencing coverage for 97.6% of target CpGs per sample, on average, and was highly reproducible between biological replicates (Supplementary Fig. 2). We identified a strongly differentially methylated region (DMR) from ~ +3.2 kb to −0.7 kb relative to the *Cd4* TSS (Fig. 2a; referred to hereafter as the TSS-proximal DMR). This DMR was hyper-methylated in $CD8^+$ cells compared to $CD4^+$ cells (Fig. 2b and Supplementary Fig. 3; 21/55 CpGs in this region exhibited >40% methylation in $CD8^+$ cells and >2x higher methylation in $CD8^+$ versus $CD4^+$ cells). Importantly, this TSS-proximal DMR straddles S4 and overlaps with the *Cd4* promoter as well as a recently identified “maturation” enhancer (adjacent to S4) required to initiate stable CD4 expression in mature helper lineage cells ([13] and P.D.I. and D.R.L., unpublished results), indicating that the DMR is likely to control functional *cis*-acting elements. We confirmed the existence of the DMR at a subset of CpGs by amplicon sequencing and methylation-sensitive restriction enzyme digests (Supplementary Figs. 4a–c). Furthermore, the cytotoxic lineage *Cd4* locus hyper-methylation was silencer-dependent, as methylation patterns in $Cd4^{S4\Delta/S4\Delta}$ $CD4^+CD8^+$ peripheral T cells closely resembled those of wild-type $CD4^+$ cells (Fig. 2b and Supplementary Fig. 4c).

To ascertain if the hyper-methylation pattern observed in $CD8^+$ T cells was stable, we also examined DNA methylation after multiple cell divisions. Wild-type $CD4^+$, wild-type $CD8^+$ and $Cd4^{S4\Delta/S4\Delta}$ $CD4^+CD8^+$ cells that had undergone >5 divisions following *in vitro* stimulation, as assessed by CFSE dilution, maintained DNA methylation patterns similar to those of their *ex vivo* precursors (Fig. 2c and Supplementary Fig. 4d). These methylation patterns were also conserved after 20 days and >10 cell divisions of expansion in lymphopenic hosts (Supplementary Fig. 4e). Thus, consistent with a direct role for DNA methylation in regulating the heritable silencing of *Cd4*, S4 dictates *Cd4* locus hyper-methylation in cytotoxic T cells, which in turn is stable through multiple cell divisions.

Cd4 locus DNA is hyper-methylated in T cell progenitors

The finding of heritable *Cd4* DNA hyper-methylation in $CD8^+$ T cells and hypo-methylation in $CD4^+$ T cells suggested that the locus undergoes selective *de novo* methylation as DP cells differentiate towards 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 hyper-methylated both at the DN3 and DP stages (Fig. 3a, Supplementary Fig. 4f–g). Most of the methylated CpGs were retained in CD8 single-positive thymocytes (CD8SP; the "SP" suffix will be used to denote maturing helper and cytotoxic cells from the thymus), while a substantial number converted to the unmethylated state in CD4SP cells (Fig. 3a). Germline S4 deletion did not affect DNA methylation in DP cells (Fig. 3b, Supplementary Fig. 4h), suggesting that while silencer-mediated activity is required to sustain DNA methylation as cells transition from DP precursors to the CD8 lineage, it is not required to maintain locus methylation in DP cells. Overall these data indicate that the *Cd4* locus becomes hyper-methylated early in T cell development, and is then selectively demethylated during specification of the $CD4^+$ T-helper lineage.

DMR methylation does not grossly alter chromatin structure

We next considered how hyper-methylation of the *Cd4* locus could impact silencing. As DNA methylation can regulate nucleosome stability and positioning in some contexts[14,15], we evaluated the possibility that *Cd4* locus DNA methylation could impact nucleosomes and, hence, chromatin compaction. Thus, we used micrococcal nuclease digestion and CATCH-seq to identify nucleosomes from diverse samples, including DN3, DP, $CD4^+$, $CD8^+$, $Cd4^{E4P\Delta/E4P\Delta}$ DP, $Cd4^{E4P\Delta/E4P\Delta}$ $CD4^+$, $Cd4^{S4\Delta/S4\Delta}$ DP, and $Cd4^{S4\Delta/S4\Delta}$ $CD8^+$. We found that the loss of nucleosomes at ~ +3.5 kb relative to the TSS, immediately downstream of the TSS-proximal DMR, correlated strongly with CD4 expression (Supplementary Fig. 5). However, DNA methylation content did not correlate well with differential nucleosome positioning, indicating that this is unlikely to be a critical function of *Cd4* locus 5mC marks in facilitating silencing.

Cd4 hypo-methylation correlates with stable CD4 expression

Cd4 hypo-methylation in helper lineage cells led us to hypothesize that the removal of 5mC marks is critical for heritable, high-level CD4 expression. To determine if this may be the case, we assessed if 5mC content was dependent on the *Cd4* proximal enhancer. $E4_P$-deficient $CD4^+$ helper T cells exhibited unstable CD4 expression upon cell division[7]. Interestingly, in naive $Cd4^{E4P\Delta/E4P\Delta}$ $CD4^+$ cells there was *Cd4* TSS-proximal DMR hyper-methylation, with 5mC content approaching that observed in mature wild-type $CD8^+$ cells (Fig. 4a) and correlating with unstable CD4 expression. Further, when we stimulated naïve $Cd4^{E4P\Delta/E4P\Delta}$ $CD4^+$ cells to proliferate *in vitro* and analyzed *Cd4* methylation patterns locus-wide in $CD4^+$ and $CD4^-$ cells after >5 cell divisions, those cells that lost CD4 expression exhibited more TSS-proximal DMR methylation (Fig. 4b). Thus, in helper T cells, *Cd4* locus hypo-methylation correlated with more stable maintenance of CD4 expression (maintaining CD4 expression after > 5 cell divisions), while hyper-methylation correlated with loss of CD4 expression. In $Cd4^{E4P\Delta/E4P\Delta}$ thymic DP cells, which lack CD4 expression[7], the *Cd4* methylation pattern was most similar to that in wild-type DN3 cells, with hyper-methylation ±1 kb from the location of $E4_P$ (Supplementary Fig. 6). Taken together, these results suggest that $E4_P$ contributes to stable CD4 expression in the helper lineage by facilitating heritable *Cd4* locus de-methylation.

Reduced DNMT1 rescues E4$_P$-KO helper cell CD4 expression

To determine if there may be a causal link between *Cd4* locus hypo-methylation and heritable CD4 expression, we interfered with DNMT1 expression in E4$_P$- deficient helper cells. We sorted naïve CD4$^+$ cells from *Dnmt1*$^{L/L}$*Cd4*-Cre$^+$, *Dnmt1*$^{L/Chip}$*Cd4*-Cre$^+$ and *Dnmt1*$^{L/L}$*Cd4*-Cre$^-$ mice, all on a *Cd4*$^{E4P\Delta/E4P\Delta}$ background, and analyzed CD4 expression at various time points after activation.

We measured CD4 protein expression, as it correlates with mRNA expression in *Cd4*$^{E4P\Delta/E4P\Delta}$ T cells[7]. At 72 h, 76.2% of *Dnmt1*-sufficient cells maintained CD4 expression (Fig. 5a). In contrast, *Dnmt1*-knockout and *Dnmt1*-hypomorphic cells exhibited higher proportions of CD4$^+$ cells (94.9% and 87.6%, respectively; Fig. 5a). CD4$^+$ *Dnmt1*-hypomorphic *Cd4*$^{E4P\Delta/E4P\Delta}$ cells also exhibited ~30% higher CD4 mean fluorescence intensity (MFI) compared to *Dnmt1*$^{+/+}$ *Cd4*$^{E4P\Delta/E4P\Delta}$ CD4$^+$ cells at 72h, indicating higher CD4 expression in the cells that remained CD4$^+$ (Fig. 5b). The increased proportion of CD4$^+$ cells (20–25% higher) and increased MFI (40–47% higher) in *Dnmt1* hypomorphic versus *Dnmt1*-sufficient cells was also observed at 96 h and 120 h (Supplementary Fig. 7a–b). Importantly, these results were not due to differences in proliferative capacity because the fraction of CD4$^+$ cells was consistently higher in *Dnmt1*-deficient cells compared to wild-type across each cell generation as measured by CFSE dilution (Fig. 5c, Supplementary Fig. 7c). Transduction of *Cd4*$^{E4P\Delta/E4P\Delta}$ CD4$^+$ T cells with retroviruses encoding *Dnmt1* shRNAs led to a similar result (Supplementary Fig. 7d–e). The results are thus consistent with E4$_P$-dependent demethylation of the *Cd4* TSS-proximal DMR being critical for establishing heritable high CD4 expression.

DMR demethylation occurs during helper lineage commitment

We next asked when DNA demethylation occurs within helper lineage cells. We examined *Cd4* TSS-proximal DMR methylation by amplicon bisulfite sequencing at different stages after positive selection and during helper lineage differentiation using a *Zbtb7b*^{GFP} reporter mouse[16]. *Zbtb7b* (which encodes ThPOK) is required for CD4-lineage commitment, and GFP is expressed from the *Zbtb7b*^{GFP} allele specifically in MHCII-selected cells[16–18]. We sorted positively selected CD69$^+$HSA$^+$CD4$^+$CD8^{lo} cells prior to lineage commitment (GFP$^-$; includes both MHCI- and MHCII-specific cells), early in helper lineage commitment (GFP^{mid}) and late in commitment (GFP^{hi}), as well as mature CD4SP cells (CD4$^+$CD8$^-$TCRβ^{hi}HSA^{lo}CD69^{lo}GFP^{hi}) (Supplementary Fig. 8a–c). Using a representative TSS-proximal DMR amplicon (Fig. 6a), we found almost complete methylation in DP (Supplementary fig. 4g), CD4$^+$CD8^{lo} GFP$^-$ and GFP^{mid} cells (Fig. 6b). DNA methylation began to decrease in GFP^{hi} CD4$^+$CD8^{lo} cells, late in commitment, and was nearly absent in CD4SP cells, consistent with our earlier results (Fig. 6b). Thus, *Cd4* locus differential methylation is initiated by the removal of DNA methylation marks late in helper lineage commitment, around the time of maximal *Zbtb7b* induction, and is completed at the mature CD4SP stage.

Zbtb7b is partially dispensable for *Cd4* demethylation

Our observation that DNA demethylation in the helper lineage occurs after *Zbtb7b* is maximally induced suggested that *Zbtb7b* may play a role in the removal of these

methylation marks. To test this hypothesis, we first assessed DNA methylation by amplicon sequencing in MHCII-selected, *Zbtb7b*$^{GFP/GFP}$ CD4^{+}CD8^{lo} and CD8SP T cell subsets. In *Zbtb7b*$^{GFP/GFP}$ mice, MHCII selected cells continue to express the GFP reporter allele, but are redirected into the cytotoxic lineage due to *Zbtb7b* deficiency[16]. We found that, despite the absence of *Zbtb7b*, MHCII-selected cells began to lose DNA methylation at the CD4^{+}CD8^{lo}GFP^{hi} stage (Fig. 6c). While *Zbtb7b*$^{GFP/GFP}$ CD8SP GFP^{+} cells exhibited higher methylation than *Zbtb7b*$^{GFP/+}$ GFP^{+} CD4SP cells, they were hypo-methylated compared to wild-type CD8SP cells (Fig. 6c,d). Thus *Zbtb7b* appears to be at least partially dispensable for intronic DMR demethylation in MHCII-selected cells. We confirmed this result by bisulfite CATCH-Seq across the *Cd4* locus in mature peripheral MHCI (GFP^{-})- and MHCII (GFP^{+})- selected *Zbtb7b*$^{GFP/GFP}$ CD8^{+} cells (Fig. 6e). GFP^{+} cells exhibited TSS-proximal DMR hypo-methylation similar to wild-type CD4^{+} cells, while MHCI-selected GFP^{-} cells exhibited hyper-methylation similar to wild-type CD8^{+} cells (Fig. 6e). Interestingly, when induced to proliferate *in vitro*, GFP^{+} *Zbtb7b*$^{GFP/GFP}$ CD8^{+} cells maintained high CD8 expression, but also initiated CD4 expression (Fig. 6f). Thus, MHCII selection can induce *Cd4* locus DNA demethylating signals even in the absence of *Zbtb7b* expression, and the resulting hypo-methylation correlates with failure to silence *Cd4* in redirected CD8^{+} cells. Moreover, *Zbtb7b* is not required for *Cd4* expression.

Cell division-independent *Cd4* locus demethylation

The removal of DNA methylation marks in the *Cd4* locus late in lineage commitment raises the question of whether demethylation is passive, by way of cell division, or active, through biochemical removal of 5mC marks. Among the 21 differentially methylated CpGs of the TSS-proximal DMR, the median DP:CD4SP CpG methylation ratio is 4.3 (mean: 27.6). Thus, passive demethylation would require an average of at least 2 cell divisions during helper lineage differentiation to effect passive demethylation, and over 9 divisions to explain the demethylation observed at some residues. Previous reports have suggested that there is no cell division between the DP and CD4SP stages of development[19], or that a minority (<20%) of CD4SP cells may divide after HSA down-regulation and within hours of thymic emigration[20,21]. Moreover, our amplicon analysis of CD4^{+}CD8^{lo} MHCII-selected cells revealed stochastic loss of DNA methylation consistent with biochemical removal or passive demethylation with some remethylation (for example, seemingly random loss of methylation at only a subset of CpGs on a given allele), rather than the mixture of all methylated and all demethylated alleles that would be indicative of purely passive demethylation resulting from cell division without maintenance methylation. To further rule out the possibility of proliferation-mediated passive demethylation, we used a CFSE labeling and thymic injection technique to determine if cells divide during differentiation into the helper lineage. We sorted CD69^{-} DP cells from CD45.2 *B2m*$^{\Delta/\Delta}$*H2-Ab1*$^{\Delta/\Delta}$ OT-II–TCR-transgenic mice, mixed these with CD45.1 CD69^{-} DP cells, stained with CFSE and injected into the thymi of CD45.1 mice that expressed MHCI and MHCII. At four days after injection, thymi were isolated and CFSE intensity and lineage differentiation were assessed by flow cytometry (Supplementary Fig. 8d–f). Among both CD45.1^{+} and CD45.2^{+} injected cells, we could identify pre–selected cells (CD69^{-} DP), positively selected (CD4^{+}CD8^{lo}CD69^{+}TCRb$^{med/hi}$) and mature CD4SP cells (CD4^{+}CD8^{-}CD69^{lo}TCRb^{hi}). All three groups expressed similarly high CFSE labeling, indicating no difference in cell divisions after injection. Importantly,

the lack of cell division was not due to loss of viability, as these cells were able to differentiate, modulating the expression of CD69, TCRβ, CD4 and CD8. Thus, without evidence of significant proliferation following positive selection, we conclude that DNA methylation is removed from the *Cd4* locus during helper lineage commitment via an active biochemical process, independent of cell division.

Ten Eleven Translocation (TET) enzyme-mediated oxidation of 5mC to 5-hydroxymethyl-cytosine (5hmC) has recently been shown to contribute to active DNA demethylation pathways[22]. To determine if TET-mediated hydroxymethylation of 5mCs in the *Cd4* locus could contribute to locus demethylation, we examined 5hmC content by T4-phage β-glucosyltransferase (T4-βGT) - mediated restriction enzyme protection-qPCR. We isolated genomic DNA from DP, $CD4^+$ and MHCII-selected $CD4^+CD8^{lo}$ cells ($CD69^+HSA^+CD4^+CD8^{lo}GFP^+$ cells from *Zbtb7b*$^{GFP/+}$ mice) and incubated the DNA with or without T4-βGT in the presence of glucose-UTP, before digestion with a restriction enzyme sensitive to modified 5hmC, MspI. T4-βGT transfers glucose-UTP specifically to 5hmC residues, blocking MspI digestion of CCGG motifs. In DP and $CD4^+$ cells, we found very little T4-βGT protection of a differentially methylated CCGG motif at +190 bp relative to the *Cd4* TSS (Fig. 7a, <20%). However, we found significantly greater T4-βGT-mediated protection of the same motif in MHCII-selected $CD4^+CD8^{lo}$ cells (~40%), consistent with TET-dependent 5hmC-mediated demethylation in differentiating helper lineage T cells. Importantly, 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 CpGs in the TSS-proximal DMR. To confirm and expand on these results, we performed oxidative-bisulfite amplicon sequencing on a group of three representative CpGs located at +1407 to +1487 relative to the TSS. While both 5mC and 5hmC are read as cytosine after bisulfite treatment, 5hmC can be oxidized by KRuO4 to 5-formylcytosine, which is then read as thymine after bisulfite treatment[23]. Thus, bisulfite treatment, with or without oxidation, can be used to differentiate 5mC from 5hmC modifications.

In DP cells, we found that all three CpGs were highly methylated (>90% 5mC/5hmC by bisulfite treatment alone), with low amounts of 5hmC at two CpGs (<20% 5hmC at CpGs 1 and 2) and moderate amounts at a third (< 50% 5hmC at CpG 3) (Fig. 7b). While overall methylation content remained similar, we observed decreased 5mC and increased 5hmC in post-selection $CD4^+CD8^{lo}$ cells compared to DP cells (Fig. 7b,c), in keeping with our previous results. It should be noted that $CD4^+CD8^{lo}$ cells are predominantly (> 2:1) MHCII selected[16]. Taken together, these results are consistent with our finding that demethylation of the TSS-proximal DMR after selection on MHCII does not require cell division, and suggest that it may be achieved enzymatically via a hydroxymethylated intermediate.

DISCUSSION

The molecular mechanism for heritable epigenetic *Cd4* silencing in cytotoxic T cells has remained elusive despite characterization of the key cis- and transacting factors required to establish the silenced state. We have shown here that DNA methyltransferases are required to maintain silencing in CD8-lineage T cells, and linked this requirement to *Cd4* locus

methylation. Conversely, our data indicate that stable CD4 expression in CD4-lineage T cells is regulated by proximal enhancer-dependent *Cd4* locus DNA demethylation. Surprisingly, the *Cd4* TSS-proximal lineage DMR was methylated early in development, in both DN and DP thymocytes, indicating that a critical function of the silencer is to antagonize demethylation in CD8-lineage cells. Finally, demethylation of the DMR in CD4-lineage thymocytes appears to involve an active enzymatic process that is likely mediated by TET-dependent oxidation of methylcytosines. Thus, heritable *Cd4* silencing versus expression is directed by a DNA demethylation switch under the control of the silencer and proximal enhancer.

Our results indicate that *Cd4* locus methylation is necessary, but not sufficient, for stable *Cd4* silencing. Indeed, DP cells express similar amounts of CD4 to helper T cells, but exhibit *Cd4* locus hyper-methylation comparable to cytotoxic lineage cells. This uncoupling of TSS-proximal DMR methylation from transcription implies that *Cd4* locus methylation/demethylation is critical for establishing heritable *Cd4* expression states rather than transcriptional activity. Still, the question remains: how do DP cells express CD4 in the face of TSS-proximal methylation? It could be due to developmental stage specific transacting factor expression. For example, Runx1 is down-regulated at the DN4 to DP transition[3,24], which could contribute to reduced S4 activity, allowing CD4 expression in spite of DNA hyper-methylation. Alternatively, or in conjunction with low Runx levels, DP cells may express yet to be identified stage-specific *Cd4* activators. Further, it should be noted that there are a handful of *Cd4* locus CpGs that are hypo-methylated in DP cells and $CD4^+$ cells relative to $CD8^+$ and DN cells; hypo-methylation of these residues in DP cells could contribute to CD4 expression in DP cells, while their *de novo* methylation in $CD8^+$ cells could contribute to silencing. Finally, it is possible that CD8 lineage cells express lineage specific factors, possibly including Runx3, that collaborate with 5mC modifications to impose a silenced state.

Our data strongly suggest that stable helper lineage CD4 expression depends on an $E4_P$-directed active DNA demethylation process in MHCII-selected thymocytes: first, $E4_P$ is required for CD4-lineage *Cd4* locus hypo-methylation; second, there is little to no cell division between the DP (hyper-methylated) and CD4SP (hypo-methylated) stages of development; third, we found 5hmC in MHCII-selected $CD4^+CD8^{lo}$ cells at TSS-proximal DMR CpGs, which are demethylated during the DP to CD4SP transition, consistent with 5mC oxidation by TET enzymes. $E4_P$ becomes dispensable in mature cells[7], in which a "maturation enhancer", putatively localized adjacent to the silencer element in intron 1 ([13] and PDI and DRL, unpublished), and within the TSS-proximal DMR, programs CD4 expression. Thus, it seems likely that the critical function of $E4_P$ is to effect TSS-proximal demethylation across the promoter and sequences flanking intronic regulatory elements, which then allows the maturation enhancer to direct CD4 expression. It is worth noting, however, that Tet enzymes can further oxidize 5hmC to 5-formylcytosine (5fC) and 5-carboxycytosine (5caC)[25–27], which are read as "unmodified" cytosines by bisulfite sequencing[23]. Thus we cannot rule out that the unmethylated CpGs identified in differentiating CD4 lineage cells represent 5fC or 5caC. Further studies will be required to

determine how demethylation and/or oxidation of cytosine residues are critical for establishing heritable CD4 expression.

Central questions raised by our study are how $E4_P$ directs demethylation in the CD4-lineage and how S4 maintains the minimally altered methylation pattern following the DP stage to CD8-lineage transition. While it is possible that Tet enzymes are recruited to $E4_P$ upon TCR signaling, we also noticed that the TSS-proximal DMR contains some 5hmC modifications in wild-type DP cells and that $E4_P$ is responsible for DP cell hypo-methylation of $E4_P$ proximal CpGs. Thus it is tempting to hypothesize that $E4_P$ recruits Tet enzymes in DP cells or earlier, to poise the 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 MHCI-selected cells? Runx3 expression is induced in MHCI-selected cells during the DP to CD8SP transition[28], and the RUNX fusion proteins found in acute myeloid leukemia have been shown to recruit DNMT proteins to target genes, presumably through an indirect mechanism[29,30]. Thus it is possible that Runx3 could recruit DNMTs to the *Cd4* locus to ensure maintenance methylation. It will be important to determine if and how TET enzymes are targeted to the *Cd4* locus in an $E4_P$-dependent manner and whether DNMT enzymes are recruited in an S4- and Runx3-dependent manner during the $CD4^+CD8^{lo}$ to $CD8^+$SP 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. The findings that the DNA methylation machinery is critical for the establishment and maintenance of silencing and that de-methylation is critical for heritable expression represent a significant advance and offer new opportunities to dissect the signaling pathways involved in thymocyte lineage choice. These results also establish *Cd4* as a unique model to understand how DNA demethylation is effected and regulated. Further investigation of how the *Cd4* locus is controlled via DNA methylation can thus provide important insights into how fully differentiated somatic cells achieve heritable states of gene expression.

METHODS

Mice

Dnmt1^{L} [31] and *Dnmt1*^{Chip} [9] mice were a kind gift from R. Jaenisch. *Dnmt3a*^{L} [32] and *Dnmt3b*^{L} [33] were from the Mutant Mouse Regional Resource Center (MMRRC). *Cd4*$^{E4P\Delta}$ [7], *Cd4*$^{S4\Delta}$, *Cd4*$^{S4\text{-}L}$ [5], and *Zbtb7b*^{GFP} [16] were previously described. WT C57BL/6, *Cd4*-Cre [8], *Ubc*-Cre$^{ER\text{-}T2}$ [34], *H2-AbI* [35], *B2m* [36], *Rag2*, *Ly5.1*, *Ly5.2* and *Tg(TcraTcrb)425Cbn* (OT-II TCR tg) mice were purchased 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^{mir} library[37] was used to generate ten retroviral pools, each comprising ~6000 shRNA clones[38]. $CD62L^+CD25^-CD8^+$ cells, isolated from spleens and lymph nodes

of *Cd4*$^{S4\text{-}L/+}$; *Ubc*-Cre^{ERT2} mice, were cultured with anti-CD3 (0.25 μg/ml) and anti-CD28 (1 μg/ml) in the presence of OH-tamoxifen (400 nM) for 16 h before transduced with the retroviral pools. After 7 days culture in the presence of IL-2 (100 U/ml), CD4-positive cells were enriched by CD4$^+$ MACS column. After 3 days, double-positive cells (CD4$^+$CD8$^+$) were sorted and their genomic DNA isolated. To identify the candidate shRNAs, the shRNA region of the transduced virus was PCR amplified, cloned and sequenced. Individual shRNAs were either obtained from the Open Biosystems library or synthesized. Two shRNA clones targeting *Dnmt1* were identified (5′-GTACACCTTTCATGATGTGAAA-3′ and 5′-TCCCGAAGATCAACTCACCAAA-3′).

Flow cytometry and sorting

Monoclonal antibodies were purchased from eBioscience or BD Bioscience. Clones used were: anti-CD4 (RM4-5), anti-CD8α (53–6.7), anti-TCRβ (H57-597), anti-HSA (M1/69), anti-CD69 (H1.2F3), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-CD45.1 (A20), anti-CD45.2 (104), CD25 (PC61) and anti-Thy1.2 (53–2.1). CFSE and e670 were obtained from Molecular Probes. After staining with antibodies and DAPI (Molecular Probes), cells were analyzed with an LSRII flow cytometer (BD Biosciences) or sorted with an Aria II (BD Biosciences). Post sort sample purity was >98%. In some cases, anti-CD4, anti-PE, anti-B220 magnetic beads (Miltenyi) were used for enrichment and depletion on the Auto-MACS platform (Miltenyi) before sorting. Flow cytometry data was analyzed using Flowjo software (Tree Star).

Cell culture

Tissue culture plates were incubated overnight with polyclonal goat anti-hamster IgG (MP biomedicals), washed 3x with PBS, and purified CD4$^+$CD8$^-$CD25$^-$CD62L$^+$CD44^{lo} or naïve CD4$^-$CD8$^+$CD25$^-$CD62L$^+$CD44^{lo} T cells were added, along with anti-CD3 (145-2C11, 0.25 μg/ml) and anti-CD28 (37.5.1, 1 μg/ml) antibodies. At day 3, cultures were supplemented with 100 U/ml recombinant IL-2 (Peprotech). For *Dnmt1* knockdown, the Dnmt1 shRNA sequences were inserted into a miR-30-based hairpin of the pMSCV-LMP vector (Open Biosystems) according to the manufacturer's instruction. Retroviruses were packaged in PlatE cells[39] by transient transfection using 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 using Purelink genomic DNA isolation kits (Invitrogen). For locus-wide bisulfite sequencing, CATCH-seq was performed as described[12], using BAC clone RP24-330J12 (BACPAC Resource Center, CHORI). For amplicon sequencing, bisulfite conversion was performed using the EpiTect Bisulfite Kit (Qiagen). Amplicons were prepared using Hot Start Ex-Taq Polymerase (TaKaRa) and the following primers: TSS-F: 5′-GGGGTATTTATTGTTTTGAGTAT-3′, TSS-R: 5′-TTTAATTTTTCAACTTCCCCAAC-3′, +1600-F: 5′-GGTTATTTGGAGTTTTTTTTTAG-3′, +1600-R: 5′-CTTCAATTCATAAACTTATTCCC-3′, and TA cloned using the Qiagen PCR cloning kit.

Bisulfite analysis of Sanger sequenced clones was performed using QUMA[40]. Oxidative bisulfite treatment was performed as previously described[41] and amplicons were analyzed as above using the following primers: +1400-F: 5′-AAGTGTTTAAAATGTGTTAATTATTG-3′, +1400-R: 5′-TTAAAAACAAAACTAAAAAAACCC-3′. T4-βGT-mediated 5mC- and 5hmC- sensitive restriction enzyme digest was performed using the EpiMARK 5-hmC and 5mC analysis kit according to the manufacturer's instructions (New England Biolabs). Quantitative PCR was performed using HotStart-IT SYBR Green qPCR Master Mix (Affymetrix-USB) and a LightCycler 480 (Roche). Percent digestion was calculated using ΔΔCt.

Nucleosome analysis

Nucleosomes were prepared as previously described[42]. Briefly, 1.1×10^7 cells were lysed in digestion buffer (50 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 0.2% Triton X-100) supplemented with protease and deacetylase inhibitors. Nuclei were then treated with 12 U Micrococcal Nuclease (Worthington Biochemical) in 135 μl digestion buffer for 5 min at 37 °C to produce >90% mono-nucleosomes. The reaction was quenched by adding EDTA to 25 mM EDTA and EGTA to 10 mM. Samples were spun for 5 min at 2500*g* and supernatants with solubilized mononucleosomes were reserved (digestion supernatants). Pelleted nuclei were then lysed twice for 1 h on ice in lysis buffer (150 μl of 1 mM Tris-HCl, pH 7.5, 0.25 mM EDTA; supplemented with Protease and deacetylase inhibitors), following gentle sonication. After removing 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank R. Jaenisch (Whitehead Institute for Biomedical Research) for mouse strains; the University of Massachusetts Medical School RNAi Core Faciility for providing shRNAs; A. Cuesta and A. Chen for technical help; and members of the laboratory for discussion. Supported by the US National Institutes of Health (R00DK091508 to J.R.H., 5 T32 CA009161-36 to M.S, and GM033977 to M.R.G.), the Jane Coffin Childs Fund (J.R.H.), the Cancer Research Institute (M.S.and P.D.I.) and the Howard Hughes Medical Institute (M.R.G. and D.R.L.).

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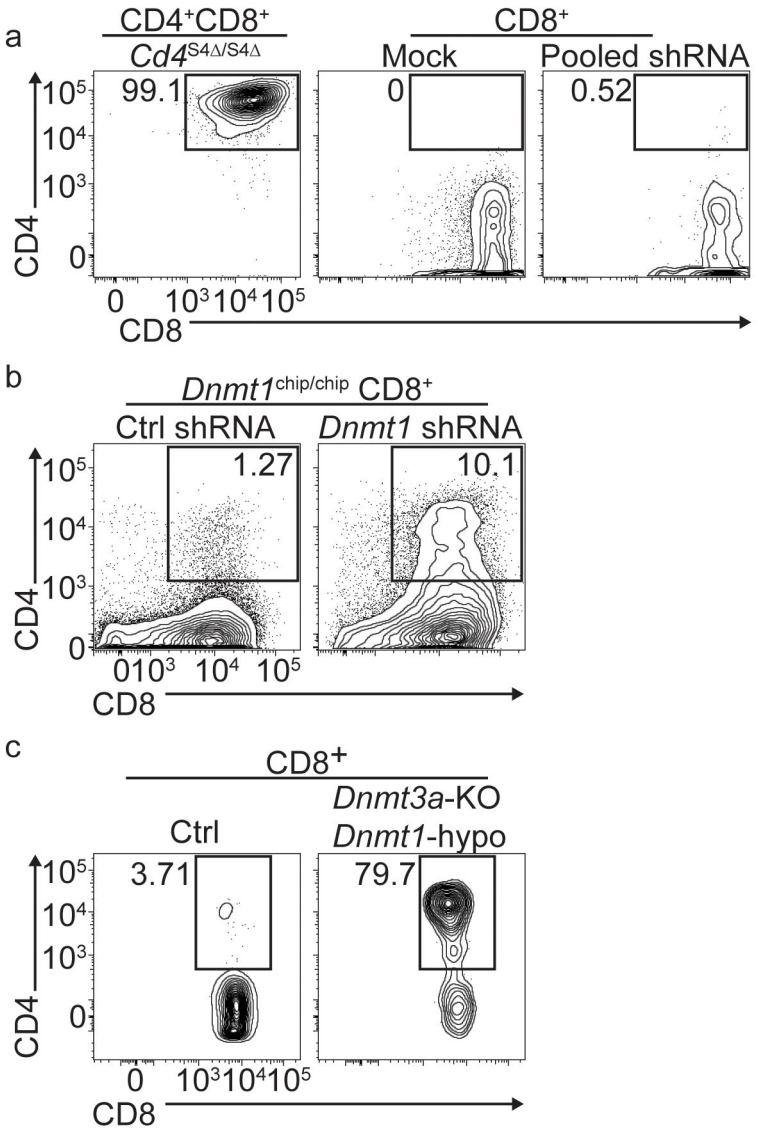

Figure 1. DNA methylation enzymes are essential for silencing CD4 expression in cytotoxic T cells

Cd4$^{S4\text{-}L}$; Ubc-Cre-ER cytotoxic cells were cultured in vitro and S4 was deleted by adding 400 nM OH-tamoxifen. (**a–c**) Flow cytometry of CD4 and CD8 expression. (**a**) Mock or pooled shRNA virus-infected cells (encoding a puromycin resistance gene) were enriched by MACS with anti-CD4 magnetic beads and cultured 4–5 more days in the presence of puromycin (2.5 μg/ml) before analysis. *Cd4*$^{S4\Delta/S4\Delta}$ $CD4^+8^+$ cells (germline S4 deletion) are shown as a staining control. Representative of 2 independent experiments. (**b**) Cytotoxic $CD4^-8^+$ cells from *Dnmt1*$^{chip/chip}$ mice were cultured *in vitro* for 5–6 days after control-ires-gfp or *Dnmt1* shRNA-ires-gfp retroviral infection. Staining is on gated GFP^+ cells. Representative of 3 independent experiments. (**c**) 1~1.5 × 10^6 peripheral $CD4^-8^+$ cells from control (*Dnmt3a*$^{L/L}$; *Dnmt3b*$^{L/+}$; *Dnmt1*$^{L/+}$) or *Dnmts*^{reduced} (*Dnmt3a*$^{L/L}$; *Dnmt3b*$^{L/+}$; *Dnmt1*$^{L/chip}$; *Cd4*-Cre$^{+/-}$) animals were adoptively transferred into Rag2-deficient mice.

$TCR\beta^{+}CD8^{+}$ cells from the periphery were analyzed 16 days after transfer. Representative of 3 independent experiments.

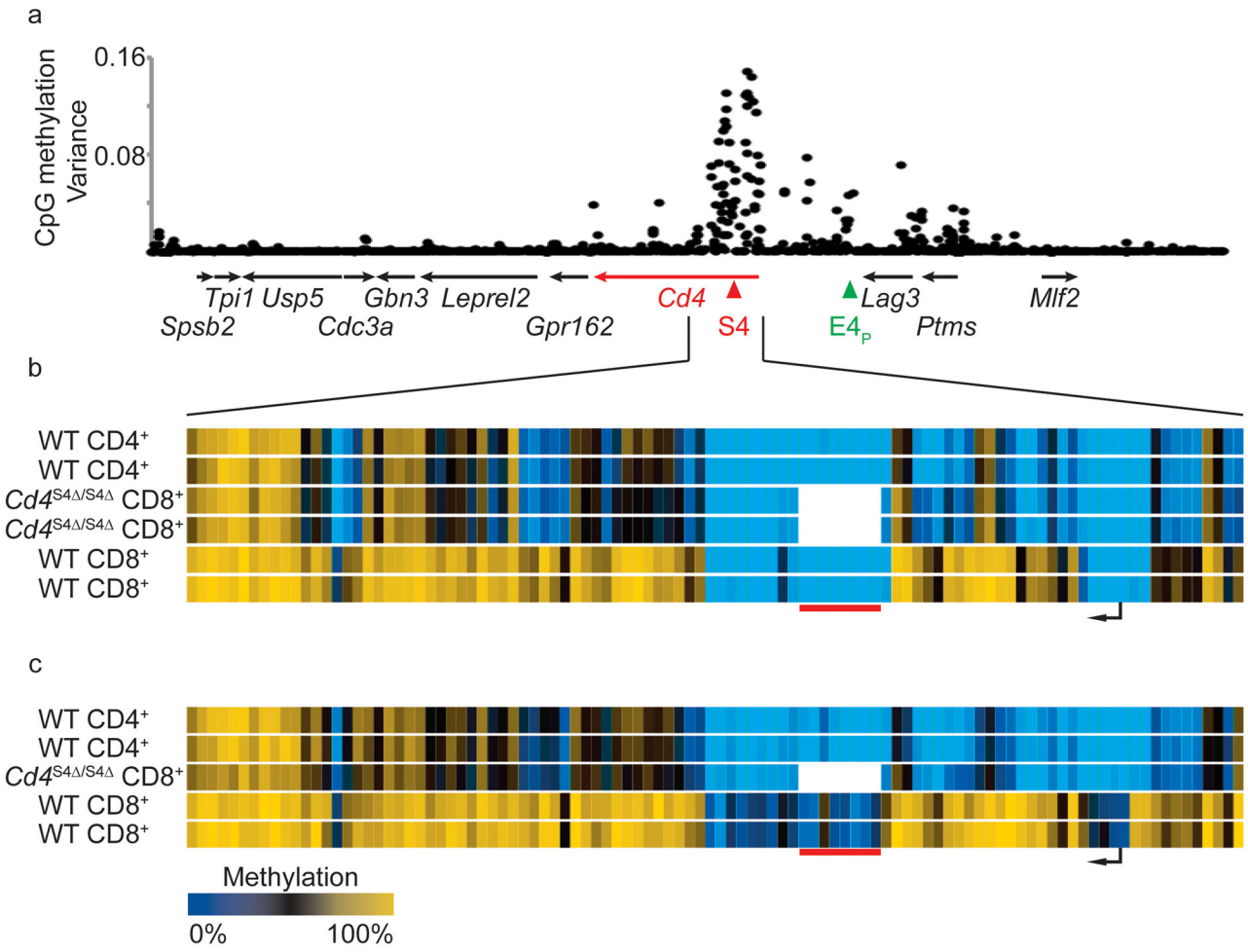

Figure 2. Silencer-dependent DMR in the first intron of *Cd4*

Biological replicates of naïve (Thy1.2$^+$CD44^{lo}CD62L$^+$CD25$^-$) WT CD4$^+$, WT CD8$^+$ and *Cd4*$^{S4\Delta/S4\Delta}$ CD4$^+$CD8$^+$ cells were isolated from LNs, and their genomic DNA was subjected to CATCH-seq (BAC-mediated enrichment, bisulfite treatment and Illumina sequencing). (**a**) Percent CpG methylation was calculated at CpGs exhibiting >30x coverage, within ~75 kb of the *Cd4* TSS (Chr6:124749635-124906460; mm9), and variance at each CpG across all samples was calculated and graphed on the *y*-axis versus genomic position on the *x*-axis. 98–98.5% of targeted CpGs were captured in each sample at >30x coverage; median CpG coverage exceeded 300x. Genes, S4 and E4$_P$ are indicated below the graph. (**b**) A heatmap depicts percent CpG methylation in WT CD4$^+$, WT CD8$^+$ and *Cd4*$^{S4\Delta S4\Delta}$ CD4$^+$8$^+$ cells for CpGs from +6200 to −669 relative to the *Cd4* TSS (Chr #6:124832027-124838896; mm9). A red line underlines CpGs in the S4 silencer (indicated by the gaps), and a black arrow indicates the *Cd4* TSS. (**c**) CFSE-labeled WT CD4$^+$, WT CD8$^+$ and *Cd4*$^{S4\Delta/S4\Delta}$ CD4$^+$CD8$^+$ cells were stimulated *in vitro* with anti-CD3, anti-CD28 and IL-2, and those cells that had undergone at least 6 divisions after 5 days were sorted for locus enrichment and high-throughput bisulfite sequencing (as above). A heatmap depicts CpG methylation percentage from +6200 to −669 relative to the *Cd4* TSS

(Chr6:124832027-124838896; mm9). Replicates were derived from two independent experiments.

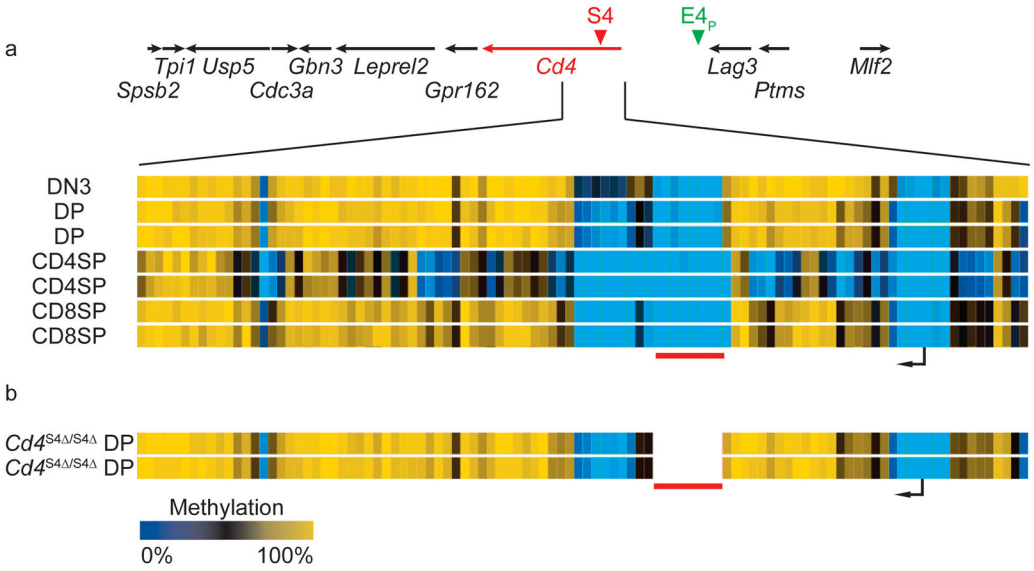

Figure 3. *Cd4* hyper-methylation in immature thymocytes

(**a**) CATCH-seq was performed on genomic DNA from sorted populations of WT thymocytes: DN3 (Thy1.2^{+}Lin^{-}CD25^{+}CD44^{-}) (n=1), DP (TCRβ^{lo}CD24^{+}CD69^{-}CD4^{+}CD8^{+}), CD4SP (TCRβ^{hi}CD24^{lo}CD69^{lo}CD4^{+}CD8–$^{-}$) and CD8SP (TCRβ^{hi}CD24^{lo}CD69^{lo}CD4^{-}CD8^{+}); the SP suffix denotes thymus-derived helper and cytotoxic T cells. A heatmap depicts CpG methylation percentage from +6200bp to −669bp relative to the *Cd4* TSS (Chr #6:124832027-124838896; mm9); Biological replicates are noted. (**b**) BAC-enrichment and bisulfite, high-throughput sequencing was performed on DP cells sorted from *Cd4*$^{S4\mathbf{\Delta}/S4\mathbf{\Delta}}$ mice. Data are displayed as in (**a**). Replicates were derived from two independent experiments.

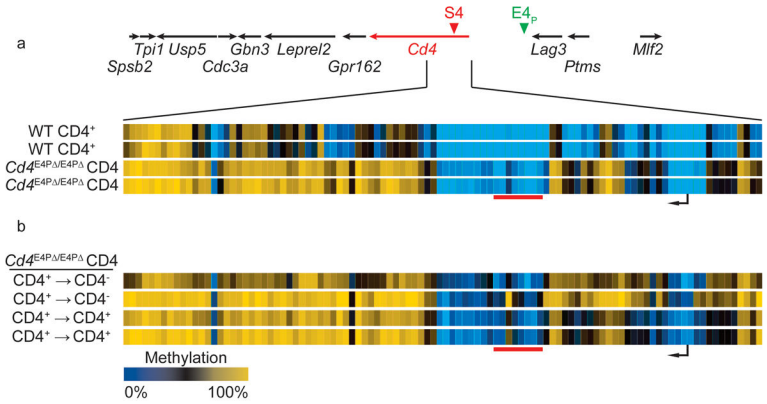

Figure 4. E4P is required for *Cd4* locus hypo-methylation in the T-helper lineage

(**a**) CATCH-seq was performed on naïve CD4$^+$ cells (Thy1.2$^+$CD25$^-$CD44^{lo}CD62L$^+$CD4$^+$) from *Cd4*$^{E4P\Delta/E4P\Delta}$; mice. A heatmap depicts percentage CpG methylation in biological replicates from +6200bp to −669bp relative to the *Cd4* TSS (Chr #6:124832027-124838896; mm9). Data from WT CD4 cells in Figure 2b are shown for comparison. (**b**) CFSE-labeled naïve CD4$^+$ *Cd4*$^{E4P\Delta/E4P\Delta}$ cells were stimulated in vitro with anti-CD3, anti-CD28 and IL-2 for 5 days. CD4$^+$ (CD4$^+$ → CD4$^+$) and CD4$^-$ (CD4$^+$ → CD4$^-$) that had undergone at least 6 divisions were sorted for CATCH-seq. Data are displayed as in (**a**). Results from 2 independent experiments are displayed.

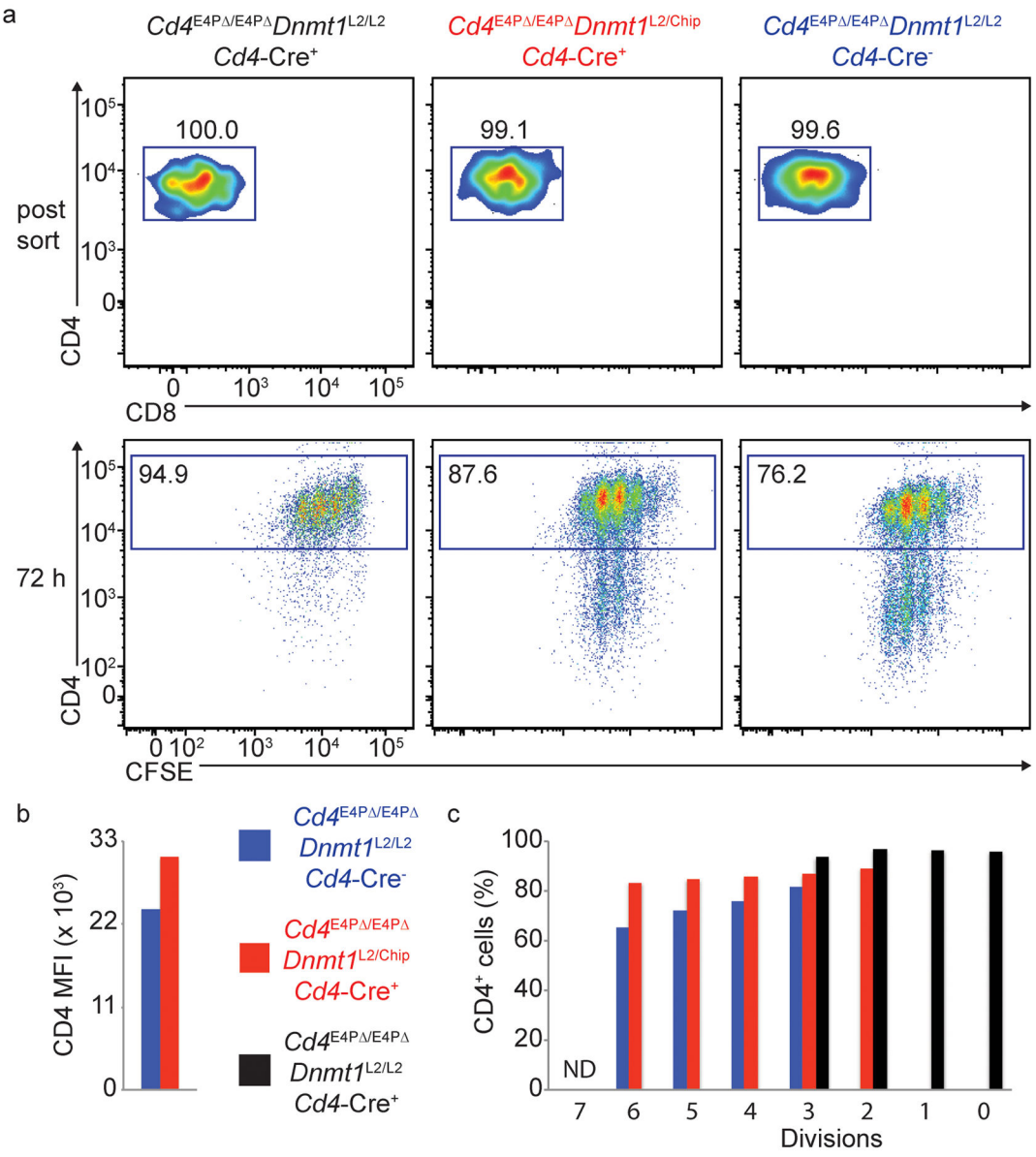

Figure 5. Reduction in Dnmt1 activity rescues CD4 expression in *Cd4*$^{E4P\Delta/E4P\Delta}$ helper T cells

CFSE-loaded naïve CD4$^+$ T cells from the indicated strains of mice were stimulated in vitro for 72 h with anti-CD3 and anti-CD28 antibodies. (**a**) Top, post-sort, pre-stimulation CD4 and CD8 expression; bottom, CD4 expression and CFSE dilution after 72 h. Percentages of CD4$^+$ cells are indicated. (**b**) Graph of CD4 MFI after 72 h activation of *Cd4*$^{E4P\Delta/E4P\Delta}$*Dnmt1*$^{L/Chip}$*Cd4-cre*$^+$ (red) and *Cd4*$^{E4P\Delta/E4P\Delta}$*Dnmt1*$^{L/L}$*Cd4*-Cre$^-$ CD4$^+$ T cells (blue) (from CD4$^+$ gates in (**a**)). *Dnmt1* knockout cells were not included as they exhibited growth defects (i.e. lower forward scatter), precluding MFI comparison (data not shown). (**c**) Percent CD4+ cells from each cell division of samples in (**a**), as tracked using dilution of CFSE fluorescence. Cells were from mice of the genotypes *Cd4*$^{E4P\Delta/E4P\Delta}$*Dnmt1*$^{L/L}$*Cd4*-Cre$^-$ (blue); *Cd4*$^{E4P\Delta/E4P\Delta}$*Dnmt1*$^{L/Chip}$*Cd4-cre*$^+$ (red); and

Cd4$^{E4P\Delta/E4P\Delta}$*Dnmt1*$^{L/L}$*Cd4*-Cre^{+} CD4^{+} (black). Results are representative of at least 4 independent experiments.

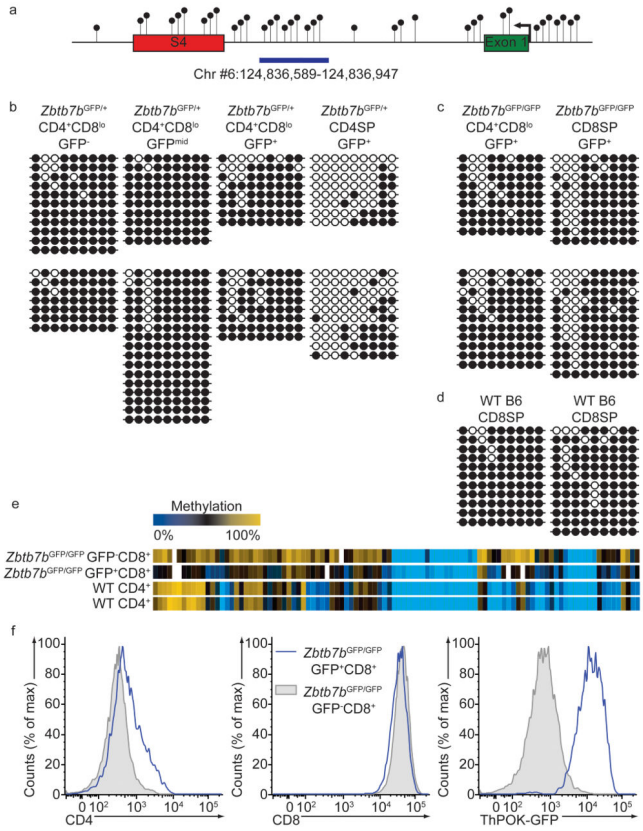

Figure 6. *Cd4* locus demethylation occurs late in helper lineage differentiation and is largely independent of *Zbtb7b*

(**a**) A *Cd4* intron 1 amplicon from the indicated sorted cells in (**b–d**) was subjected to bisulfite sequencing: (**b**) GFP^{-}, GFP^{mid} or GFP^{hi} CD4^{+}CD8^{lo} (also: CD69^{+}HSA^{hi}TCRβ^{+}) and GFP^{+} CD4SP (CD69^{-}HSA^{lo}TCRb^{+}) thymocytes from *Zbtb7b*$^{GFP/+}$ mice, (**c**) GFP^{hi} CD4^{+}CD8^{lo} and GFP^{+} CD8SP thymocytes from *Zbtb7b*$^{GFP/GFP}$ mice. (**d**) WT CD8SP thymocytes (CD69^{-}HSA^{lo}TCRb^{+}). Filled circles indicate methylated CpGs and empty circles indicate unmethylated CpGs. Biological replicates from independent experiments are shown for (**b–d**). (**e**) CATCH-seq was performed on GFP^{+} (MHCII selected) and GFP^{-} (MHCI selected) T cells from the lymph nodes of *Zbtb7b*$^{GFP/GFP}$ mice (n=1). The heatmap depicts the percentage CpG methylation from +6200bp to −669bp relative to the Cd4 TSS (Chr #6:124832027-124838896; mm9). Data from WT CD4 cells in Figure 2b, are shown for comparison. (**f**) *Zbtb7b*$^{GFP/GFP}$ GFP^{+} and GFP^{-} lymph node CD8+ cells were stimulated in vitro for 3d with anti-CD3 and anti-CD28, and then analyzed for CD4, CD8 and GFP expression; results are representative of 2 independent experiments.

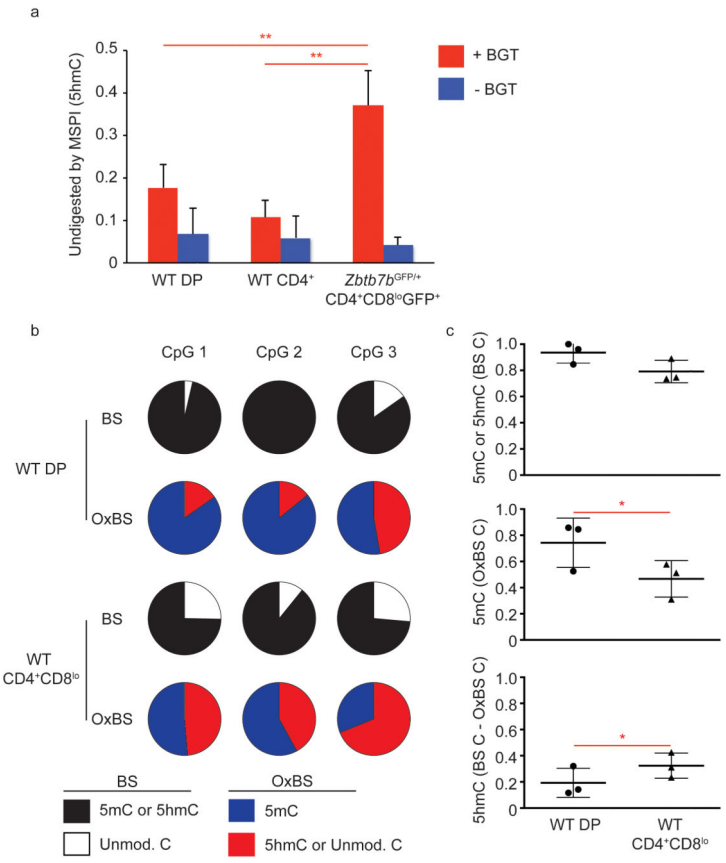

Figure 7. *Cd4* TSS-proximal hydroxymethylation in MHCII-selected CD4$^+$CD8^{lo} cells

(**a**) Genomic DNA from DP, naïve CD4$^+$ and MHCII-selected CD4$^+$CD8^{lo} cells (CD69$^+$HSA$^+$CD4$^+$CD8^{lo}GFP$^+$ cells from *Zbtb7b*$^{GFP/+}$ mice) was incubated with Uridine Diphosphate Glucose (UDG), with or without β-glucosyltransferase (βGT), before digestion with MspI. T4-βGT transfers UDG specifically to 5hmC residues, blocking MspI digestion of CCGG motifs. MspI digestion (at Chr6:124838036; +191bp relative to the *Cd4* TSS) was assessed by qPCR comparison to undigested DNA. As a loading control, values were normalized to an adjacent amplicon without a MspI site. The mean and SD of 3 (CD4$^+$) or 4 (DP and CD4$^+$CD8^{lo}) independent biological samples analyzed in two independent experiments are shown; *P* values (unpaired student's *t*-test) below 0.05 (*) and 0.01 (**) are noted. (**b–c**) Genomic DNA from DP and CD4$^+$CD8^{lo} cells was subjected to bisulfite amplicon sequencing (BS) or KRuO4-oxidation followed by bisulfite amplicon sequencing (OxBS). (**b**) Pie charts represent the mean percentage of the indicated cytosine modifications at three CpGs located from +1407 to +1487 relative to the *Cd4* TSS (CpG 1 = Chr #6:124836820; CpG 2 = Chr #6:124836779; CpG 3 = Chr #6:124836740), in DP (n=2) and CD4$^+$CD8^{lo} cells (n=2); Data were combined from 2 independent experiments: 4–13 amplicons (BS) or 11–17 amplicons (OxBS) were analyzed for each sample. (**c**) The mean and SD of the indicated modifications at the 3 CpGs in (**b**) were graphed for DP and CD4$^+$CD8^{lo} cells (circles and triangles represent the measurements for individual DP and CD4$^+$CD8^{lo} CpGs, respectively). 5hmC levels were calculated by subtracting the percentage

"C" after OxBS treatment (5mC only) from the percentage "C" after BS treatment (5mC + 5hmC). *P* values (paired student's *t*-test) below 0.05 (*) and 0.01 (**) are noted.