The histone chaperone CAF-1 cooperates with the DNA methyltransferases to maintain *Cd4* silencing in cytotoxic T cells

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The transcriptional repression of alternative lineage genes is critical for cell fate commitment. Mechanisms by which locus-specific gene silencing is initiated and heritably maintained during cell division are not clearly understood. To study the maintenance of silent gene states, we investigated how the *Cd4* gene is stably repressed in CD8$^+$ T cells. Through CRISPR and shRNA screening, we identified the histone chaperone CAF-1 as a critical component for *Cd4* repression. We found that the large subunit of CAF-1, Chaf1a, requires the N-terminal KER domain to associate with the histone deacetylases HDAC1/2 and the histone demethylase LSD1, enzymes that also participate in *Cd4* silencing. When CAF-1 was lacking, *Cd4* derepression was markedly enhanced in the absence of the de novo DNA methyltransferase Dnmt3a but not the maintenance DNA methyltransferase Dnmt1. In contrast to Dnmt1, Dnmt3a deficiency did not significantly alter levels of DNA methylation at the *Cd4* locus. Instead, Dnmt3a deficiency sensitized CD8$^+$ T cells to *Cd4* derepression mediated by compromised functions of histone-modifying factors, including the enzymes associated with CAF-1. Thus, we propose that the heritable silencing of the *Cd4* gene in CD8$^+$ T cells exploits cooperative functions among the DNA methyltransferases, CAF-1, and histone-modifying enzymes.

The establishment of distinct gene expression programs in given lineages and their maintenance through subsequent cell divisions are important features of animal development. These properties are readily observed in hematopoiesis, during which stem cells give rise to numerous cell lineages of diverse functions. Among cells of the adaptive immune system, the development of the major subsets of T cells—the CD4$^+$ helper or regulatory T cells and the CD8$^+$ cytotoxic T cells—provides an example of a binary decision followed by maintenance of cell lineage properties. Expression of the coreceptors CD4 and CD8 is coupled to the unique transcriptional programs of these functionally distinct lineages. In addition, CD4 and CD8 expression mark distinct stages of maturation in the thymus, and investigation of their transcriptional regulation has been widely used to understand lineage commitment and cellular memory (Taniuchi 2016). Early thymic precursors are double-negative (DN) for both CD4 and CD8, and, following selection for productive rearrangements of the *Tcrb* locus (β selection), there is up-regulation of both CD4 and CD8 (double-positive [DP] stage). During the DP stage, T cells rearrange the genes encoding TCRα, and those cells with heterodimeric TCRs that interact with self-peptide bound to MHC class II and class I undergo selection and differentiate into either CD4 single-positive (CD4SP) or CD8 single-positive (CD8SP) cells, respectively, that subsequently exit the thymus as mature helper/regulatory and cytotoxic T cells.

CD4 expression during development is achieved by the coordinated activity of multiple enhancers and a silencer at the *Cd4* locus (for review, see Issuree et al. 2017). The *Cd4* silencer (S4) resides in the first intron of the *Cd4* gene, and its activity is mediated by direct binding of Runx1 and Runx3 transcription factors (Sawada et al. 1994; Taniuchi et al. 2002; Setoguchi et al. 2008). Deletion of S4 or Runx complexes during T-cell development leads to *Cd4* derepression in DN thymocytes and a failure to establish silencing in CD8SP cells (Zou et al. 2001; Setoguchi et al. 2008). In contrast, deletion of S4 or deficiency of Runx factors in mature CD8^{+} T cells does not result in upregulation of CD4, consistent with earlier establishment of heritable epigenetic marks that silence the locus (Zou et al. 2001; Shan et al. 2017). Thus, S4 mediates the establishment of silencing during T-cell development but is later dispensable for its maintenance in CD8^{+} T cells. Recently, some of the molecular processes responsible for maintaining *Cd4* repression in proliferating CD8^{+} T cells were described as involving DNA methylation and covalent histone modifications (Sellars et al. 2015; Verbaro et al. 2018). In mammals, DNA methylation occurs at cytosines predominantly in the context of the CpG dinucleotide and is generally associated with transcriptional repression, although the extent of its instructive role in gene silencing remains an area of investigation (Bestor et al. 2015; Schübeler 2015). The DNA methyltransferase (DNMT) enzymes critical in establishing methylation patterns during embryogenesis and gametogenesis are Dnmt3a, Dnmt3b, and Dnmt3c and are often referred to as the de novo DNA methyltransferases. In contrast, Dnmt1 has been ascribed the predominant role in maintaining methylation after DNA replication and is referred to as the maintenance DNA methyltransferase (Li and Zhang 2014; Barau et al. 2016). Dnmt1 is recruited to the replication fork through interactions with PCNA, the sliding clamp, and Uhrf1, which binds to hemimethylated DNA (Smith and Meissner 2013).

In eukaryotes, genomic DNA is organized into chromatin, in which the basic subunit is the nucleosome. The nucleosome is composed of ~147 bp of dsDNA wrapped around a histone octamer core particle comprised of one tetramer of H3–H4 flanked by two dimers of H2A–H2B, and these core particles are connected through linker DNA. Posttranslational modifications (PTMs) of nucleosomal histones provide marks that can regulate gene expression (Bannister and Kouzarides 2011). Examples include H3/H4 acetylation and H3K4 methylation, which are generally associated with activated or permissive transcription states, and H3K9 and H3K27 methylation, which are generally associated with gene silencing (Jenuwein and Allis 2001). These dynamic changes have been involved in regulating cell fate decision and maintenance of cell identity (Yadav et al. 2018). Deficiencies in the DNA methyltransferases or the histone methyltransferase G9a, which catalyzes H3K9 monomethylation and dimethylation (H3K9me1/2), can cause *Cd4* derepression in CD8^{+} T cells during cell proliferation (Sellars et al. 2015; Verbaro et al. 2018). Furthermore, DNA methylation is regulated during T-cell development. CD4SP cells undergo demethylation at the *Cd4* locus, relative to DP and CD8SP cells, in a differentially methylated region (DMR) spanning +3.2 to −0.7 kb relative to the *Cd4* transcriptional start site (TSS) (Sellars et al. 2015). Interestingly, DP thymocytes harbor active histone modifications at the *Cd4* locus despite having hypermethylated DNA. This configuration suggests cross-talk and hierarchical interactions between these regulatory pathways (Yu et al. 2008; Chong et al. 2010; Sellars et al. 2015). Thus, the study of the *Cd4* locus represents an attractive system to understand how DNA methylation and other epigenetic modifications may cooperate to regulate transcriptional memory, an integral component of vertebrate development (Cedar and Bergman 2009).

In this study, we sought to identify additional factors involved in the maintenance of *Cd4* silencing and asked how they cooperate with the DNA methylation machinery to confer heritability of the repressed state. Through CRISPR and shRNA screening, we identified the histone chaperone complex CAF-1 as another factor required for *Cd4* silencing. CAF-1 is a conserved heterotrimeric complex comprised of Chaf1a, Chaf1b, and Rbbp4 that promotes H3–H4 deposition during DNA replication in S phase (Smith and Stillman 1989; Kaufman et al. 1995). The CAF-1 complex has well-established roles in the maintenance of heterochromatin and cell identity, but its function in regulating gene expression in primary somatic cells and its coordination with DNA methylation in gene silencing have been largely unexplored (Tchenio et al. 2001; Krawitz et al. 2002; Houlard et al. 2006; Dohke et al. 2008; Quivy et al. 2008; Huang et al. 2010; Heyd et al. 2011; Cheloufi et al. 2015; Ishiuchi et al. 2015; Roelens et al. 2017). Mechanistically, we found that the CAF-1 subunit Chaf1a associates with the histone deacetylases (HDACs) HDAC1/2 and the histone demethylase LSD1, enzymes that counteract histone marks associated with active transcription. CAF-1 deficiency also induced accumulation of "active" histone marks at the *Cd4* locus in CD8^{+} T cells. In assessing concomitant Dnmt1 and CAF-1 deficiency, we found that CD8^{+} T cells exhibited a modest increase in *Cd4* derepression with hypomethylation of the *Cd4* locus. Remarkably, CAF-1 combined with Dnmt3a deficiency did not cause significant demethylation of the *Cd4* locus but markedly increased *Cd4* derepression. Furthermore, loss of Dnmt3a led to *Cd4* derepression that was significantly enhanced by pharmacological inhibition of HDACs or deficiency of CAF-1-associated histone-modifying enzymes. Thus, CAF-1 and the DNA methyltransferases cooperate to maintain *Cd4* silencing and thereby contribute to CD8^{+} T-cell function and lineage integrity.

Results

The histone chaperone CAF-1 is required for the maintenance of Cd4 repression in CD8^{+} T cells

We used CD8^{+} T cells to screen for factors involved in the maintenance of *Cd4* silencing. We targeted 648 genes associated with chromatin with a retroviral shRNA library consisting of 5048 shRNA clones. Transduced cells in

the CD8^{+}CD4^{+} and CD8^{+}CD4^{−} fractions were sorted, and shRNA clones enriched in the former set of cells were identified by DNA sequencing (Supplemental Fig. S1A, B). Retroviral shRNA clones corresponding to subunits of the histone chaperone complex CAF-1 (Supplemental Fig. S1C, blue) and *Dnmt1* (Supplemental Fig. S1C, orange) were identified in the screen and found to up-regulate CD4 expression to varying degrees in validation experiments (Supplemental Fig. S1C). To further validate these identified chromatin regulators, we performed in parallel a candidate-based *Cd4* derepression screen with retroviral sgRNA transduction of CD8^{+} T cells from *Cas9*[Tg] mice (Fig. 1A; Supplemental Table 1). Multiple guide RNAs (gRNAs) for CAF-1 subunits (Fig. 1A, blue) and the maintenance DNA methylation machinery (Fig. 1A, orange) induced CD4 up-regulation relative to negative control guides targeting genes lowly expressed in CD8^{+} T cells, the olfactory receptor *Olfr2*, and the CD4^{+} lineage transcription factor *Thpok* (Fig. 1A, black). We validated these initial findings by repeating the sgRNA targeting of all three CAF-1 subunits (*Chaf1a*, *Chaf1b*, and *Rbbp4*) (Fig. 1B, blue), which reproducibly up-regulated CD4 expression similarly to the previously reported mediator of *Cd4* silencing, *Dnmt1* (Fig. 1B, orange; Sellars et al. 2015). Although CAF-1 has a well-established role in the proliferation and viability of mammalian cells, the limited time frame of the sgRNA cultures and CAF-1 protein half-life were likely mitigating factors allowing us to target these essential genes.

To begin dissecting potential interactions of CAF-1 and the DNA methyltransferases in *Cd4* silencing, we next assayed the effects of combined targeting of both sets of factors. CAF-1 knockdown (KD) resulted in enhanced *Cd4* derepression in CD8^{+} T cells from mice hypomorphic for Dnmt1 (*Dnmt1*[chip/+]) (Tucker et al. 1996) compared with wild-type (WT) mice (Fig. 1C). When cells were concomitantly deficient in CAF-1 and the de novo DNA methyltransferases (*Cd4*-Cre; *Dnmt3a*[f/f] *Dnmt3b*[f/f], referred to here as 3A3B knockout [KO]), we found a markedly enhanced effect, with the majority of repression due to Dnmt3a (Supplemental Fig. S1D). We further confirmed the critical role of Dnmt3a by targeting the CAF-1 subunits with sgRNAs transduced into *Cas9*[Tg] CD8^{+} T cells deficient for Dnmt3a (*Cd4*-Cre; *Dnmt3a*[f/f], referred to here as 3A KO), observing an enhancement similar to that seen with the shRNA KD (Fig. 1D). Thus, both CAF-1 and the DNA methyltransferases are essential for the maintenance of *Cd4* silencing in cytotoxic T cells.

Dnmt3a deficiency enhances the repressive effects mediated by other replication fork-associated factors

From our candidate-based sgRNA screening, we also identified several other factors involved in *Cd4* silencing—notably, the chromatin remodeling enzyme Smarca5 and the replicative helicase component Mcm2. Both factors have been found to be associated with replication foci, and Mcm2 has been implicated recently to have an additional role as a H3–H4 histone chaperone (Poot et al. 2004; Huang et al. 2015). We validated their functions by gene targeting

using CRISPR and observed marked enhancement with Dnmt3a deficiency (Supplemental Fig. S2A,B). These data suggest that replication fork-associated factors in addition to CAF-1 also participate in *Cd4* silencing and cooperate with Dnmt3a to bolster repressive function.

Dnmt3a represses Cd4 in mature CD8^{+} T cells, and its absence sensitizes to HDAC inhibitor-mediated derepression

Because we observed significantly greater *Cd4* derepression by combining deficiencies of CAF-1 and Dnmt3a, we further explored their relationship in gene silencing. Previously, we reported that several CpGs acquire methylation during the differentiation of DP thymocytes into CD8SP cells in the thymus (Sellars et al. 2015). This raised the possibility that thymic deficiency of Dnmt3a (achieved by *Dnmt3a* deletion with *Cd4*-Cre) might lead to latent defects in silencing that manifest during T-cell activation. Thus, we decided to test Dnmt3a for a postthymic maintenance role in *Cd4* repression by targeting the *Dnmt3a* and control *Dnmt1* genes with electroporated sgRNA amplicons in naïve nonproliferating CD8^{+} T cells from *Cas9*[Tg] mice (Supplemental Fig. S3A). The electroporated cells were then activated and transduced with retroviral sgRNA targeting *Chaf1a*. Targeting *Dnmt3a* resulted in substantial *Cd4* derepression as compared with targeting *Dnmt1* (Supplemental Fig. S3A). We further tested for a postthymic role for Dnmt3a by ectopically expressing it in 3A KO CD8^{+} T cells and found that it could rescue *Cd4* repression after *Chaf1a* KD (Fig. 2A). This depended on an intact DNA methyltransferase domain, as the catalytic-dead Dnmt3a_V712G mutant was unable to rescue silencing (Zhang et al. 2018). These results indicate that Dnmt3a is required for silencing in mature proliferating CD8^{+} T cells.

Evidence for a repressive role of Dnmt3a in mature CD8^{+} T cells led us to speculate that it might also participate in DNA methylation maintenance with Dnmt1, as has been suggested in postmitotic neurons and other contexts (Feng et al. 2010; Hervouet et al. 2018). Therefore, we next assessed levels of DNA methylation by bisulfite PCR amplicon sequencing of regions within the *Cd4* DMR. In agreement with our previous work, we observed demethylation from the DP thymocyte to the CD4^{+} T-cell stage and persistent DNA methylation within CD8^{+} T cells at the TSS and +1600 bp downstream in the first intron, adjacent to the regulatory elements S4 and the maturation enhancer (E4M) (Supplemental Fig. S3B,C; Sellars et al. 2015; Issuree et al. 2018; Kojo et al. 2018). We next assessed DNA methylation after CRISPR targeting of *Dnmt3a* by electroporating sgRNAs into *Cas9*[Tg] CD8^{+} T cells and could not detect an appreciable difference in methylation within the +1600 region of the DMR as compared with the *Olfr2* targeted control in sorted CD8^{+}CD4^{+} cells or in the bulk population that was combined with Chaf1a deficiency (Fig. 2B; Supplemental Fig. S3D).

To systematically address changes in DNA methylation and rule out the possibility that methylation changes occur at other important sites within the *Cd4* locus, we
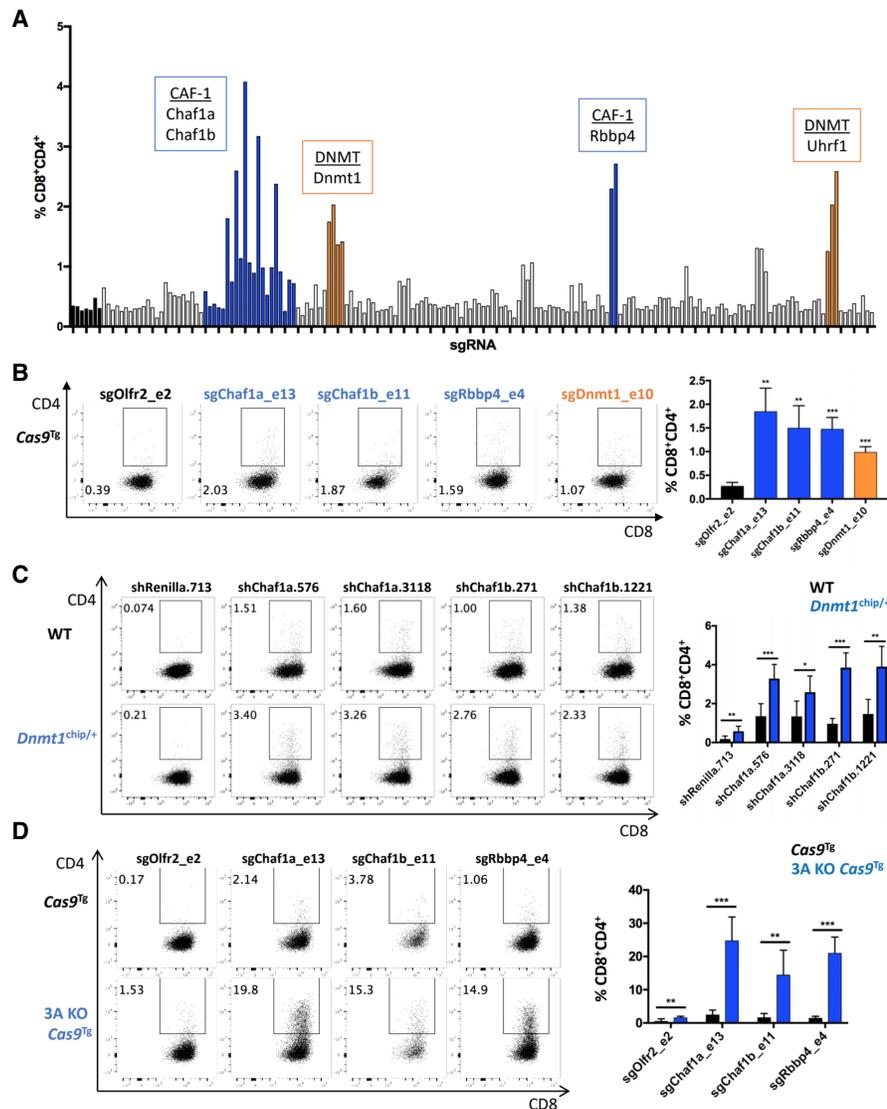
Figure 1. CRISPR screening identifies histone chaperone CAF-1 as regulator of *Cd4* silencing in mature CD8+ T cells. (*A*) One-hundred-eighty-two retroviral sgRNA constructs targeting 141 genes were transduced into *Cas9*[Tg] CD8+ T cells to screen for *Cd4* derepression. Negative controls (*Olfr2* and *Thpok*) are shown in black, CAF-1 subunits are shown in blue, and maintenance DNA methyltransferase components (*Dnmt1* and *Uhrf1*) are shown in orange. (*B*) Validation of candidate genes from *A* by sgRNA transduction of *Cas9*[Tg] CD8+ T cells. Data are means ± SD. $n = 4$. (**) $P < 0.01$; (***) $P < 0.001$ by Student's *t*-test. (*C*) CD8+ T cells from wild-type (WT) or *Dnmt1*[Chip/+] mice were isolated and transduced with the indicated shRNAs. Data are means ± SD. $n = 5$. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$ by Student's *t*-test. (*D*) Dnmt3a-deficient or Dnmt3a-sufficient *Cas9*[Tg] CD8+ T cells were transduced with the indicated sgRNAs targeting CAF-1. Data are means ± SD. $n = 4$. (**) $P < 0.01$; (***) $P < 0.001$ by Student's *t*-test.

performed whole-genome methylation analysis on DNA from CD8+CD4+ T cells after targeting *Dnmt3a* or *Dnmt1* with electroporated sgRNA (Supplemental Fig. S3E). Consistent with the bisulfite PCR amplicon sequencing, there was little change in DNA methylation along the entirety of the *Cd4* locus in CD8+CD4+ T cells after Dnmt3a targeting, with total levels of DNA methylation similar to those in *Olfr2* targeted control CD8+CD4− T cells. In contrast, *Dnmt1* targeting induced widespread hypomethylation in CD8+CD4+ T cells.

The contrasting roles of Dnmt1 and Dnmt3a in controlling DNA methylation in mature T cells led us to hypothesize that disabling the two classes of DNA methyltransferases would lead to enhanced *Cd4* derepression. Consistent with our previous results (Sellars et al. 2015), in 3A3B KO CD8+ T cells, there was marked *Cd4* derepression after *Dnmt1* KD, suggesting that widespread DNA methylation remained an intact mechanism of repression at the *Cd4* locus (Supplemental Fig. S4). Thus, given the lack of gross defects in DNA methylation after loss of Dnmt3a and the retained sensitivity to Dnmt1 inhibition in 3A3B KO cells, we explored whether Dnmt3a may influence *Cd4* derepression through the effects of histone modifications, such as histone acetylation and
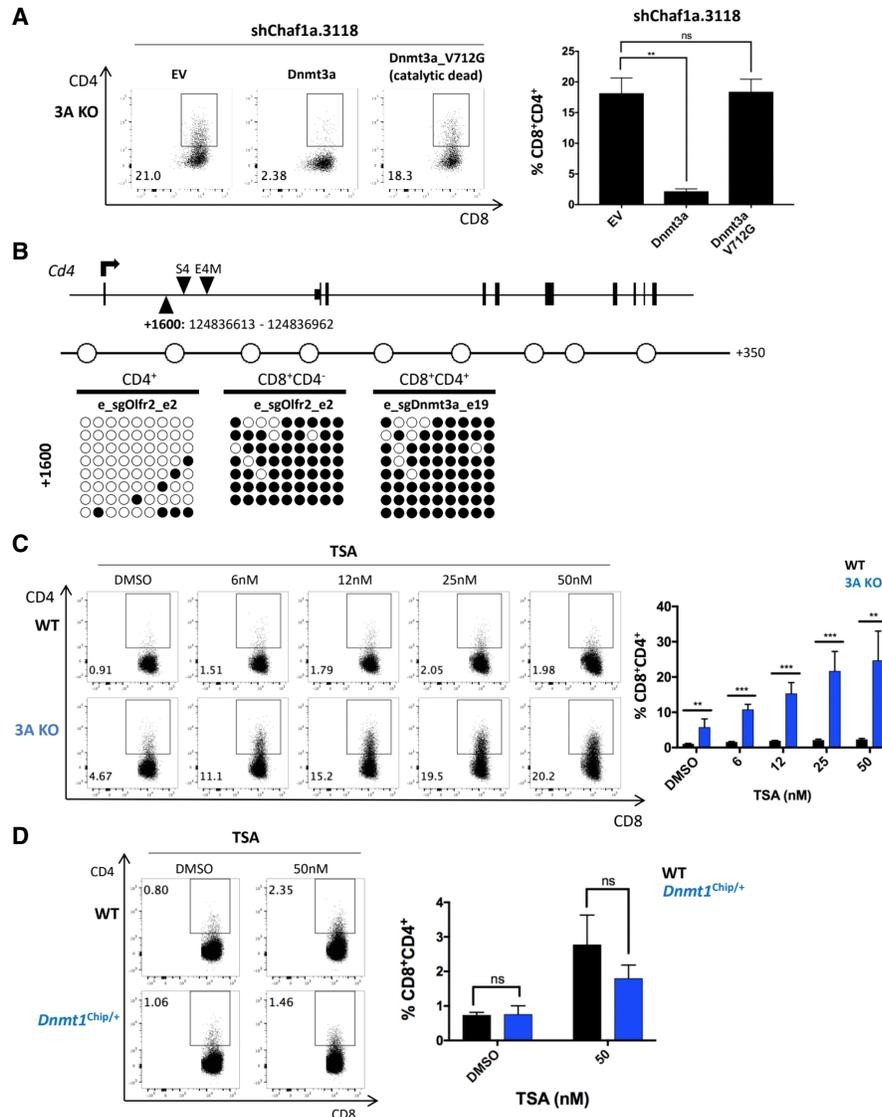
Figure 2. Dnmt3a deficiency in mature CD8$^+$ T cells potentiates *Cd4* derepression mediated by CAF-1 deficiency and activating histone modifications. (*A*) 3A KO CD8$^+$ T cells were cotransduced with retroviral shRNA targeting Chaf1a together with retroviral overexpression vectors encoding WT or mutant Dnmt3A. Data are means ± SD. *n* = 3. (ns) Not statistically significant; (**) *P* < 0.01 by Student's *t*-test. (*B*) T cells from *Cas9*^{Tg} mice were electroporated with the indicated amplicons (e_sgRNA) and subsequently sorted according to CD4 expression. Genomic DNA was isolated for bisulfite PCR sequencing of the +1600 region within the DMR. The schematic shows the relative density of CpG dinucleotides assessed (open circles), and arrowheads indicate the +1600 region and regulatory elements S4 and E4M. (*C*) WT or 3A KO CD8$^+$ T cells were treated with increasing doses of pan-HDAC inhibitor trichostatin A (TSA). Data are means ± SD. *n* = 4. (**) *P* < 0.01; (***) *P* < 0.001 by Student's *t*-test. (*D*) *Dnmt1*$^{Chip/+}$ or WT CD8$^+$ T cells were treated with the HDAC inhibitor TSA and assayed for CD4 up-regulation. Data are means ± SD. *n* ≥ 4. (ns) Not statistically significant by Student's *t*-test.

deacetylation, prominent mechanisms of gene regulation (Jenuwein and Allis 2001). Treatment of 3A KO CD8$^+$ T cells with the pan-HDAC inhibitor trichostatin A (TSA) potently enhanced *Cd4* derepression (Fig. 2C). In contrast, there was only a modest effect of TSA on CD4 expression in WT CD8$^+$ T cells, and this was not further accentuated by *Dnmt1* deficiency (Fig. 2D). Taken together, these data support a role for Dnmt3a in repressing *Cd4* expression at least in part through sensitizing

cells to changes in histone modifications, such as histone acetylation.

CAF-1 and Dnmt1 regulate gene expression through distinct mechanisms

To further elucidate the connection between the DNA methyltransferases and CAF-1 in maintaining *Cd4* silencing, we compared DNA methylation and chromatin

accessibility in cells in which *Cd4* derepression was achieved through KD of either Chaf1a or Dnmt1. As expected from the results following inactivation of both Chaf1a and Dnmt3a (Supplemental Fig. S3D), there was no appreciable change in demethylation after Chaf1a KD even in sorted cells that up-regulated CD4 expression (Fig. 3A; Supplemental Fig. S5A). The absence of DNA demethylation following CAF-1 deficiency was consistent with previous reports in *Arabidopsis thaliana* and a murine fibroblast cell line (Tchenio et al. 2001; Schönrock et al. 2006). In contrast, after Dnmt1 KD, we observed marked DNA demethylation, with greater demethylation correlating with *Cd4* derepression.

Since we did not detect changes in DNA methylation after CAF-1 deficiency, we used ATAC-seq to assess changes in chromatin accessibility, which was shown previously to be influenced by CAF-1 (Tchenio et al. 2001; Cheloufi et al. 2015). In the first intron of the *Cd4* locus, CD4+ T cells have increased accessibility at E4M and decreased accessibility at S4 (Supplemental Fig. S5B). However, after Chaf1a KD in CD8+ T cells, we did not detect significant changes in accessibility in the first intron of cells in which *Cd4* was derepressed, as compared with CD8+ T cells treated with control shRNA (Fig. 3B). In contrast, Dnmt1 KD led to reduced accessibility at the silencer element in CD8+ T cells, which resembled chromatin accessibility in CD4+ T cells (Fig. 3B). Consistent with a role for DNA methylation in altering chromatin accessibility at regulatory elements S4 and E4M, there was no notable change in CD8+CD4+ T cells after Dnmt3a sgRNA targeting (Supplemental Fig. S5C). It should be noted that there was a modest increase in accessibility at the *Cd4* promoter after Dnmt3a targeting, which may reflect an alternative pathway to activation.

Because of the differences in DNA methylation and chromatin accessibility at the *Cd4* locus following Chaf1a versus Dnmt1 KD, we sought to determine whether these changes were representative of genome-wide effects under each of these conditions. After sorting cells based on *Cd4* derepression, we found that the CD8+ T-cell transcriptomes clustered according to shRNA treatment rather than CD4 expression status. The majority of differentially expressed genes relative to control shRenilla transduction were unique, indicating primarily nonoverlapping roles for both Chaf1a and Dnmt1 in CD8+ T cells (Fig. 3C). Chaf1a deficiency dysregulated more genes than Dnmt1, suggesting that DNA methylation had more limited effects on gene regulation relative to chromatin-mediated activities of CAF-1. Notably, the populations with CD4 derepression did not up-regulate characteristic genes of the CD4+ lineage (Fig. 3D). Furthermore, ATAC-seq samples clustered by shRNA treatment rather than CD4 expression and separately from CD4+ T helper cells (Fig. 3E). This suggests that the derepression of the *Cd4* gene itself did not confer a change in accessibility sufficient to uniquely separate this population. Taken together, these results indicate that the CD8+ T cells that derepress *Cd4* do not undergo lineage conversion.

Both Dnmt1 deficiency and Chaf1a deficiency were involved primarily in gene repression (Supplemental Fig. S6A). To gain more insight into the underlying mechanism, we next compared genome-wide the magnitude of change in gene expression with the corresponding magnitude of change in chromatin accessibility. We focused on genes that were divergently expressed following Chaf1a versus Dnmt1 KD and found that gene accessibility and expression correlated to a higher degree after deficiency of Dnmt1 compared with CAF-1, albeit with modest R^2 values (Supplemental Fig. S6B). The modest correlation was likely due to several confounding factors, including small percentages of cells undergoing stochastic gene derepression, changes in accessibility of distal enhancers that could not be assigned to specific genetic loci, and complex forms of regulation occurring in *trans*. Notably, in both sets of data, there were examples of significant changes in gene expression with and without corresponding changes in accessibility (Supplemental Fig. S6C).

Characterization of Chaf1a domains involved in Cd4 silencing

To gain more insight into the mechanism used by CAF-1 to silence *Cd4*, we performed complementation assays with shRNA-resistant Flag-tagged *Chaf1a* constructs. We cotransduced retroviral shRNA and *Chaf1a* cDNA constructs into 3A KO CD8+ T cells to provide a larger signal to noise ratio. Full-length (FL) Chaf1a rescued *Cd4* repression efficiently relative to empty vector (EV) (Fig. 4A). Previous studies identified N-terminal truncations of Chaf1a that were capable of rescuing essential CAF-1 function in vivo (Hoek et al. 2011). Other N-terminal truncations were shown to act as dominant-negative mutants that impaired gene repression and heterochromatin formation in mammalian cells (Tchenio et al. 2001; Reese et al. 2003). We assessed two N-terminal truncation mutants, referred to as T1 (amino acids 338–911) and T10 (amino acids 409–911) (Fig. 4B). T1 rescued Chaf1a deficiency equally well to FL Chaf1a (Fig. 4C). The rescue effect was specific to Chaf1a deficiency, as Dnmt1 KD was not rescued by ectopic expression of T1 or FL. Furthermore, T1 required the ED domain (glutamate-rich and aspartate-rich region) (Fig. 4B, green box), as its internal deletion abrogated rescue function without affecting its expression level (Supplemental Fig. S7A,B). Analysis of construct T10 showed that it possessed dominant-negative activity, as it enhanced *Cd4* derepression in the context of Renilla or Dnmt1 KD (Fig. 4C) but did not further enhance derepression upon Chaf1a KD. The T10 protein, which lacks the KER domain, had a greatly reduced ability to interact with PCNA and histone H3 in transfected Plat-E cells but retained its association with CHAF1B relative to the FL and T1 molecules (Fig. 4D; Supplemental Fig. S7C).

Chaf1a contains two PCNA-interacting peptide (PIP) motifs: PIP1 and PIP2. The N-terminal PIP1, absent in T1, was not required for rescue. PIP2 was shown to be essential for nucleosome assembly in vitro and for CAF-1 function in vivo (Rolef Ben-Shahar et al. 2009). Furthermore, its disruption was reported to result in interference with gene repression in yeast and in loss of PCNA binding (Krawitz et al. 2002). To determine the effect of disrupting
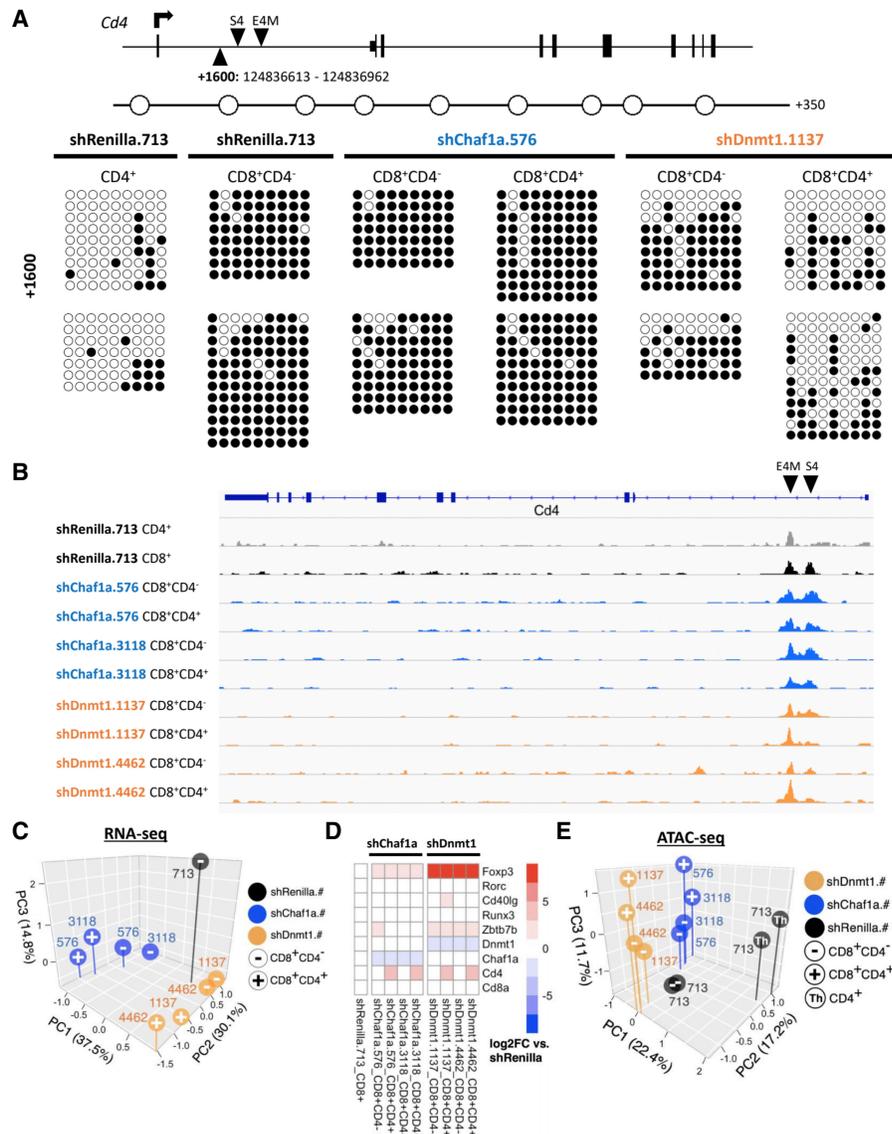
Figure 3. CAF-1 deficiency induces *Cd4* expression without significant changes in DNA methylation or chromatin accessibility. (*A*) Bisulfite PCR amplicon sequencing of the *Cd4* +1600 region was performed after shRNA transduction and sorting of T cells of the indicated phenotype (two independent biological replicates are shown). The schematic shows the relative density of CpG dinucleotides assessed (open circles), and arrowheads indicate the +1600 region and regulatory elements S4 and E4M. (*B*) ATAC-seq tracks of the *Cd4* locus of the indicated T-cell samples. The regulatory elements E4M and S4 are indicated by downward arrowheads. (*C*) Principal component analysis (PCA) of RNA-seq from CD8⁺ T cells transduced with the indicated shRNAs and sorted based on the indicated phenotypes. (*D*) Heat map depicting expression levels of selected CD4⁺ lineage genes in the indicated samples. (*E*) PCA of ATAC-seq from CD8⁺ T cells transduced with the indicated shRNAs and sorted based on the indicated phenotypes.

the Chaf1a–PCNA interaction on *Cd4* derepression, we assessed the function of a PIP2 mutation within the T1 backbone—T1-PIP2 (Chaf1a_F428A)—that was unable to rescue silencing (data not shown). This molecule showed dominant-negative activity in CD8⁺ T cells, conferring increased CD4 expression relative to EV or T1 (Fig. 4E). Similarly to T10, the T1-PIP2 protein retained association with the other subunits of CAF-1, suggesting that dominant-negative Chaf1a mutants may sequester endogenous CAF-1 subunits away from the replication fork, as

hypothesized previously (Supplemental Fig. S7D; Tchenio et al. 2001; Ye et al. 2003). Interestingly, the T1-PIP2 construct had a less severe phenotype than T10 (which was also evident in WT CD8⁺ T cells) despite a similar loss in PCNA binding (Supplemental Fig. S7E). This suggests that additional functions of the KER region, such as potentially mediating histone binding (Supplemental Fig. S7C) or its recently reported role in DNA binding, might also be relevant for *Cd4* silencing (Sauer et al. 2017). Thus, from our structure–function analysis, we conclude that
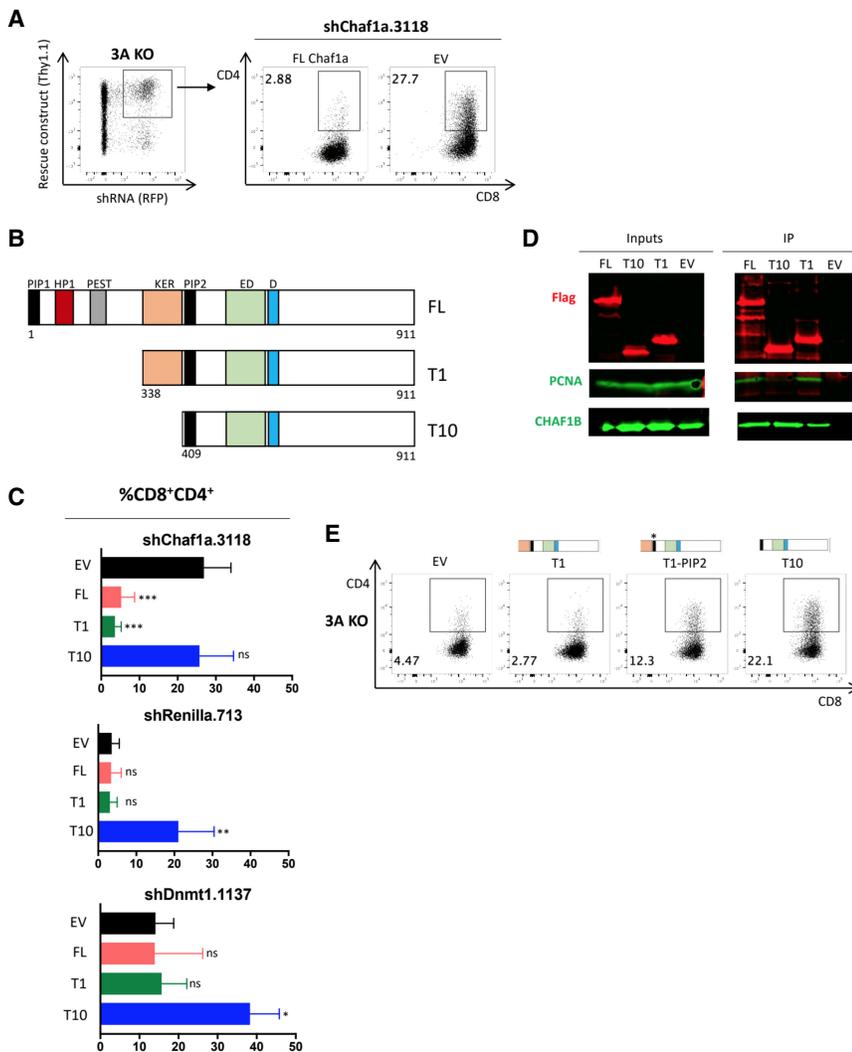
Figure 4. PCNA binding and the KER region are required for Chaf1a-mediated repression of *Cd4*. (*A*) 3A KO CD8+ T cells were cotransduced with shRNA targeting *Chaf1a* (shChaf1a.3118) plus shRNA-resistant FL Chaf1a or EV control, and doubly transduced gated cells were evaluated for expression of CD4. (*B*) Schematic of domain organization of mouse FL Chaf1a protein, the rescue truncation (T1), and the dominant-negative truncation (T10). Flag tag was added to the C termini of all constructs (not depicted). Colored regions indicate the PCNA-interacting peptide 1 (PIP1; black), heterochromatin-associated protein 1 domain (red), PEST domain (gray), KER domain (orange), PIP2 (black), ED domain (green), and dimerization domain ("D," blue). (*C*) 3A KO CD8+ T cells were cotransduced with the indicated retroviral shRNA and rescue constructs and assessed for CD4 expression. Data are means ± SD. $n \geq$ 4. (ns) Not statistically significant; (*) $P <$ 0.05; (**) $P < 0.01$; (***) $P < 0.001$ by Student's *t*-test. (*D*) Immunoprecipitation of Flag-tagged *Chaf1a* constructs transfected into Plat-E cells and probed for the ability to pull down PCNA and CHAF1B. (*E*) CD4 expression after transduction of the indicated *Chaf1a* constructs into 3A KO CD8+ T cells. Data are representative of two independent experiments.

both the PCNA interaction and the KER region are essential for Chaf1a to repress *Cd4*.

Chaf1a associates with histone-modifying enzymes that are also required for the maintenance of Cd4 silencing

CAF-1 is reported to contribute to the maintenance of heterochromatin integrity through its association with factors and enzymatic activities enriched at heterochromatin loci (Reese et al. 2003; Sarraf and Stancheva 2004; Heyd et al. 2011; Yang et al. 2015; Cheloufi and Hochedlinger 2017). Using the rescue and dominant-negative constructs T1 and T10, respectively, we screened for binding partners by mass spectrometry in transfected Plat-E cells. The top hits for T1 and T10 after subtracting the EV negative control pull-down were CHAF1B and RBBP4, the other two subunits of the CAF-1 complex (Fig. 5A; Supplemental Table 2). Interestingly, T1, but not T10, associated with HDAC1 and HDAC2 and the histone H3K4 demethylase LSD1, proteins within transcriptional corepressor complexes that are involved in removing active histone

modifications (Delcuve et al. 2012). These protein associations were reported previously with the FL Chaf1a, and we confirmed their interactions with the T1 construct in transfected Plat-E cells (Fig. 5B; Heyd et al. 2011; Yang et al. 2015). These data suggest that the KER region is required for the interaction of Chaf1a with these epigenetic modifiers. Furthermore, the association of HDACs and LSD1 with T1 did not occur indirectly through PCNA, as it was observed even with the PCNA-binding mutant T1-PIP2.

We next assessed whether these histone-modifying enzymes function in the maintenance of *Cd4* silencing by targeting their genes with sgRNAs in Dnmt3a-sufficient or 3A KO CD8+ T cells. Modest CD4 up-regulation occurred after targeting *Hdac1* or *Lsd1* in Dnmt3a-sufficient cells, and this was substantially enhanced in the absence of Dnmt3a (Fig. 5C). Similarly, pharmacological inhibition of LSD1 showed Dnmt3a-dependent enhancement (Supplemental Fig. S8). Finally, to determine whether CAF-1 deficiency may affect the histone modification status imposed by HDACs and LSD1, we assessed by ChIP-qPCR for the presence of H3K9ac and H3K4me2 as well as for

H3K4me3, a modification that marks active promoters (Fig. 5D). After Chaf1a KD, CD8^{+} T cells that had up-regulated CD4 also had increased levels of H3K4me3, H3K9ac, and H3K4me2. Thus, CAF-1 deficiency leads to increased levels in active histone modifications at the *Cd4* locus. These data indicate that CAF-1 enables removal of "active" or "permissive" marks at the *Cd4* locus, potentially by promoting the deacetylase and demethylase activities of HDACs and LSD1, respectively.

Discussion

The discovery that sustained *Cd4* silencing in mature T cells is independent from the DNA sequence required for its establishment led to the proposal that epigenetic factors were involved in maintaining the repressed state (Zou et al. 2001). Both DNA and histone modification-based mechanisms of *Cd4* silencing have been invoked, but their potential cooperation and cross-talk were not explored (Sellars et al. 2015; Verbaro et al. 2018). In this study, we used shRNA and sgRNA screening to identify chromatin-associated factors that repress *Cd4* expression in mature CD8^{+} T cells. We further characterized their relationships with the DNA methyltransferases, enzymes identified previously as mediating the maintenance of *Cd4* silencing (Henson et al. 2014; Sellars et al. 2015). Although we focused on the replication-coupled H3–H4 histone chaperone CAF-1, we also found additional *Cd4* repressors in our candidate sgRNA screen, including the chromatin remodeling enzyme *Smarca5* and the replicative helicase component *Mcm2*. Like CAF-1, the repressive capacity of these factors in silencing *Cd4* was potentiated by Dnmt3a, suggesting a general role for replication fork-associated factors and the de novo DNA methyltransferases in gene regulation. Future study of additional factors that participate in *Cd4* repression based on our candidate screening may help in further understanding the relationship between chromatin-based and DNA-based mechanisms of heritable transcriptional silencing.

Investigation of combined Dnmt1 and CAF-1 deficiency revealed modest but reproducible enhancement of *Cd4* derepression. CAF-1 and Dnmt1 deficiencies showed differences with respect to alterations in chromatin accessibility and DNA methylation at the *Cd4* locus. These distinct effects of Dnmt1 and CAF-1 deficiencies were mirrored in their unique gene expression profiles. Interestingly, in contrast to thymic deficiency in the absence of histone methyltransferase G9a, another maintenance factor in *Cd4* silencing, we did not observe a CD4^{+} lineage-like phenotype in CD8^{+} T cells that had up-regulated CD4 (Verbaro et al. 2018). This may reflect differences in the studies, as we targeted the genes in mature cells after thymic maturation. Conditional deletion of CAF-1 components during development followed by transcriptomic analysis will help determine any potential relationship between CAF-1 and G9a activities.

In contrast to Chaf1a KD or Dnmt3a targeting, reduced Dnmt1 resulted in hypomethylation of the *Cd4* DMR, whose extent correlated with CD4 up-regulation. The intermediate DNA methylation level after Dnmt1 KD seen in CD4^{−} cells may indicate that there is a threshold of DNA demethylation that is required for *Cd4* derepression. Alternatively, targeted CD4^{−} and CD4^{+} cells may be equally prone to derepression, and further demethylation in the derepressed fraction may be a consequence of transcription, as noted in other contexts (Bestor et al. 2015). Interestingly, our recent study found that failure to demethylate the *Cd4* locus during CD4^{+} T-cell development due to TET enzyme deficiency resulted in unstable CD4 expression in activated helper T cells but did not affect expression of CD4 in CD4SP thymocytes (Issuree et al. 2018). Thus, DNA demethylation at the *Cd4* locus may not be involved in establishing CD4 expression but rather may serve to stabilize its expression state during cell division.

Dnmt1 KD also induced *Cd4* locus accessibility resembling that in CD4^{+} helper T cells, and this effect was more pronounced in the CD4^{+} fraction in a fashion analogous to the increased DNA demethylation observed in this population. Although the functional consequences of DNA methylation altering the chromatin landscape of S4 and E4M are unclear, nucleosome positioning in the first intron of *Cd4* was found to correlate with its expression status (Sellars et al. 2015). Additionally, such changes in chromatin accessibility may modulate the stability of *Cd4* derepression despite our previous finding that this region is dispensable for heritable silencing (Zou et al. 2001). In this regard, testing *Cd4* expression levels over time after induction of CAF-1 or Dnmt1 deficiency will be informative. Interestingly, Dnmt3a deficiency did not incurr either significant demethylation or notable changes in chromatin accessibility, lending further support to the hypothesis that the accessibility in the first intron of the *Cd4* locus is sensitive to methylation status.

In our analysis of *Cd4* silencing, we found strong synergies in *Cd4* derepression when Dnmt3 and CAF-1 deficiencies were combined. We found previously that several cytosine residues acquired de novo methylation—likely due to Dnmt3 enzymatic activity—during progression from the DP to the CD8SP stage (Sellars et al. 2015). Here, we observed that Dnmt3 activity was also required for *Cd4* silencing in peripheral CD8^{+} T cells. Unexpectedly, we could not observe significant changes in DNA methylation in mature CD8^{+} T cells that had up-regulated *Cd4* expression after targeting *Dnmt3a* or in cells that were doubly deficient with Chaf1a. Furthermore, Dnmt1 KD in 3A3B KO cells potently up-regulated CD4 expression, a finding consistent with the interpretation that, in the absence of the Dnmt3 enzymes, methylated CpGs were still available to be passively demethylated, with consequent *Cd4* derepression. However, as the catalytic domain of Dnmt3a was required for rescuing 3A KO CD8^{+} T cells, there may be individual CpG residues affected that hold disproportionate importance for *Cd4* repression (although full-genome methylation sequencing revealed no obvious loss of methylation at any residues within the locus), or there may be indirect effects from the up-regulation of
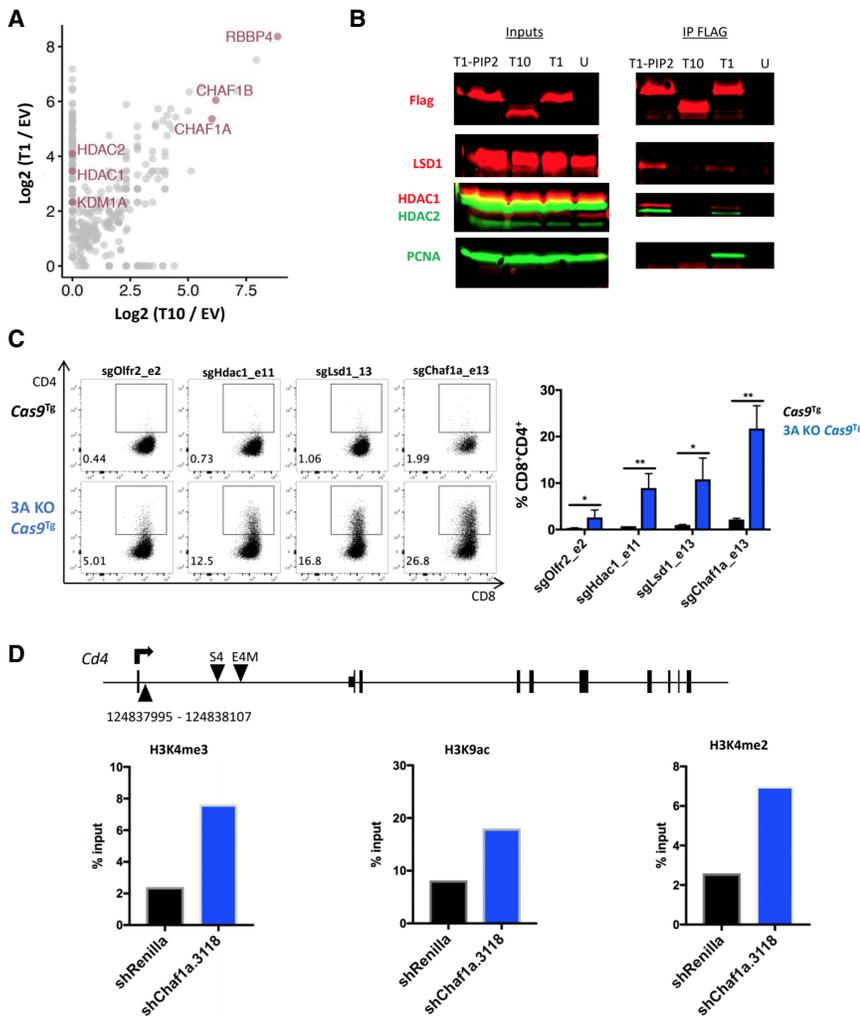
Figure 5. Chaf1a associates with histone-modifying enzymes that are required for the maintenance of *Cd4* silencing in CD8+ T cells. (*A*) Immunoprecipitation-mass spectrometry results from anti-Flag pull-down of extracts from Plat-E cells transfected with EV and T1 and T10 constructs. T1 and T10 were plotted with EV subtracted as background. CAF-1 components shared between T1 and T10 and repressive histone-modifying enzymes specific for the T1 rescue construct are highlighted in maroon. (*B*) Flag pull-down from transfected Plat-E cells, blotted for the histone-modifying enzymes shown in *A*. (*C*) Retroviral sgRNA constructs were transduced into $Cas9^{Tg}$ or 3A KO $Cas9^{Tg}$ CD8+ T cells, and CD4 expression was assessed. Data are means ± SD. $n \geq 4$. (*) $P < 0.05$; (**) $P < 0.01$ by Student's *t*-test. (*D*). Histone modifications were assessed in sorted CD8+CD4+ T cells adjacent to the *Cd4* TSS after shRNA KD of Chaf1a (shChaf1a.3118) or in sorted CD8+CD4− T cells after control transduction (shRenilla.713). Data are averaged from two biological replicates.

other factors that can promote *Cd4* expression. Alternatively, the mutation of the Dnmt3a catalytic domain may alter its interaction with binding partners, as has been reported with DNMT3A R882 mutants, and this may subsequently influence the heritable silencing of *Cd4* independently of changes in DNA methylation (Koya et al. 2016).

The specific sensitization of Dnmt3a-deficient CD8+ T cells to *Cd4* derepression upon treatment with histone-modifying drugs or genetic targeting of Chaf1a-associated histone-modifying enzymes suggests a functional coupling of Dnmt3a to the regulation of histone modifications. Although we focused on the removal or prevention of activating histone modifications, another possibility is that Dnmt3a facilitates repressive histone marks that counteract the activating effects of histone acetylation and H3K4 methylation. Also, as is the case with DNA methylation, the extent of a transcriptionally instructive role for histone modifications remains uncertain (Ptashne 2013). In light of the increased H3K4 methylation and H3K9 acetylation induced by CAF-1 deficiency and strong correlation with CD4 induction, the *Cd4* locus may be a good model to test for potential instructive and

heritable functions of these modifications, such as by conditionally and specifically targeting histone-modifying enzymes, now made feasible with gene-editing tools.

To gain more insight into the mechanism by which CAF-1 participates in gene silencing, we identified domains of the protein that are critical for its repressive function. We identified N-terminal truncations that either can rescue or have dominant-negative function in the repression of *Cd4*. This allowed us to use mass spectrometry to identify binding partners specific to our rescue-competent and dominant-negative mutants. We found that the KER region, but not the PIP2 motif, was required for the association of Chaf1a with multiple histone-modifying enzymes, including the HDACs HDAC1/2 and histone demethylase LSD1. CRISPR targeting of these molecules led to *Cd4* up-regulation that was significantly potentiated by Dnmt3a deficiency, emphasizing the connection of Dnmt3a to the regulation of histone modifications. However, compared with the targeting of *Chaf1a*, we could not achieve similar levels of *Cd4* derepression when targeting *Lsd1* or *Hdac1* individually. This likely reflects in part the combinatorial functions of these and potentially other CAF-1-associated

enzymes. Deficiency of CAF-1-mediated nucleosome assembly may also affect gene repression in an indirect manner that is not mutually exclusive to its role in recruiting histone-modifying enzymes. For example, defective nucleosome assembly by CAF-1 could lead to compensatory recruitment of the histone variant H3.3, which has been suggested to participate in gene activation (Ray-Gallet et al. 2011; Weber and Henikoff 2014). As reported previously for gene repression in yeast, we found that association with PCNA was critical for CAF-1-mediated *Cd4* repression, specifically through the internal PIP2 motif, which is required for recruitment to replication foci and replication-coupled nucleosome assembly (Krawitz et al. 2002; Rolef Ben-Shahar et al. 2009; Zhang et al. 2016). Similarly, we found that the KER region, a predicted coiled-coil domain, was also required for Chaf1a-mediated silencing. This may be due to its roles in nucleosome assembly and in mediating DNA binding, as shown recently for the yeast homolog of Chaf1a (Sauer et al. 2017). In future studies, it will be important to distinguish KER-mediated recruitment of histone-modifying enzymes from its function in chromatin assembly by using mutants specifically disabling each activity.

The *Cd4* locus serves as a model to investigate the heritable repression of alternative lineage genes in primary somatic cells. In this study, we found a novel connection between CAF-1, repressive histone-modifying enzymes, and DNA methylation in the repression of the alternative lineage gene *Cd4* in CD8^{+} T cells. We delineated maintenance roles in *Cd4* silencing for both Dnmt3a and Dnmt1 and found unique synergy between Dnmt3a and CAF-1 that involved the inhibition of gene activation-associated histone acetylation and methylation. Thus, we propose that chromatin-based mechanisms of silencing through CAF-1 and histone-modifying enzymes collaborate with the DNMTs in the repression of *Cd4* (Supplemental Fig. S9). Given the evolutionarily conserved role of CAF-1 in the maintenance of heterochromatin and cell identity, further clarifying how it functions at the crossroads with other epigenetic pathways will likely provide important insight into gene regulation, differentiation, and development.

Materials and methods

Mice

Cd4-Cre, *Dnmt1*^chip, *Dnmt3a*^f/f , and *Dnmt3b*^f/f mouse strains were described previously (Lee et al. 2001; Sellars et al. 2015). Mice harboring the Cas9 transgene (Cas9^Tg) were purchased from Jackson Laboratories. The Cas9 strains with constitutive (stock no. 026179) or conditional (stock no. 026175 and crossed to *Cd4*-Cre) expression of Cas9 were used interchangeably. Experiments were performed on male and female mice age 6–12 wk. All mice were maintained in the animal facility at the Skirball Institute. Experiments with animals were conducted according to approved protocols for the New York University Institutional Animal Care and Usage Committee.

Primers and antibodies

All primers and antibodies are listed in Supplemental Table 3.

Plasmids and rescue experiments

The retroviral sgRNA expression vector pSIN-U6-sgRNA-EF1as-Thy1.1-P2A-Neo was generated by mutating a pSIN vector backbone and the 5′ LTR to remove BbsI sites and assembling a hU6-BbsI-filler-BbsI-tracr EF1as-Thy1.1-P2A-Neo cassette downstream from the packaging signal, and shRNA vectors LENG (MSCV-miRE-pgk-Neo-IRES-GFP) and LENC (MSCV-miRE-pgk-Neo-IRES-mCherry) have been described previously (Fellmann et al. 2013). For rescue experiments, murine *Chaf1a* cDNA or *Dnmt3a* cDNA was cloned into an MSCV-IRES-Thy1.1 retroviral vector. The catalytic-dead Dnmt3a mutant was made using Gibson (New England Biolabs), where the V712 codon, equivalent to human V716, was mutated (Zhang et al. 2018). Flag tag (amino acids DYKDDDDK) was added to the C termini and N termini of Chaf1a and Dnmt3a rescue constructs, respectively. For the T1-ED construct, the ED domain (amino acids 518–613) was replaced with a Gly–Ser–Gly–Ser linker. Rescue constructs were transduced into 3A KO CD8^{+} T cells interchangeably with 3A KO *Cas9*^Tg CD8^{+} T cells.

In vitro culture of CD8^{+} T cells and retroviral transduction

T cells were isolated from mouse spleen and lymph nodes and purified by positive selection with MACS anti-CD8 magnetic beads according to the manufacturer's protocol (Miltenyi Biotech) or by cell sorting for naïve cells (TCRβ^{+}CD8^{+}CD25^{−}CD62L^{+}CD44^{−}) on FACSAria (BD Biosciences). For T-cell activation, wells were precoated with 1 mg/mL goat antihamster IgG at 1:40 dilution (MP Biomedicals). T cells were resuspended in T-cell medium (TCM): RPMI (Fisher Scientific) supplemented with 10% FBS (Atlanta Biologicals), 4 mM glutamine, 50 μM β-mercaptoethanol, and 20 mM HEPES. For activation, TCM was further supplemented with 1 μg/mL anti-CD3 (Bio X Cell) and 1 μg/mL anti-CD28 (Bio X Cell). After overnight activation, T cells were transduced by spin infection with retroviral particles with 10 μg/mL polybrene (Sigma). Spin infection was performed by centrifugation at 850*g* for 1.5 h at 32°C. T cells were kept in TCM supplemented with 100 U/mL human IL-2 (Peprotech) for 4–6 d prior to analysis for CD4 expression.

shRNA screen

An shRNA library focused on 625 epigenetic regulators was cloned into the LENG vector and used to generate a retroviral pool for infection (Cheloufi et al. 2015). CD8^{+} T cells were isolated from the spleens and lymph nodes of C57BL/6 (WT) mice by MACS anti-CD8 magnetic beads (Miltenyi), activated as described above, and cultured an additional 6 d prior to cell sorting. DNA was extracted by standard methods, and barcoded sequencing libraries were prepared using a nested PCR strategy with AmpliTAQ Gold polymerase (Thermo Fisher): 20 PCR cycles using primers MA389 and MA391 followed by a secondary PCR for 25 cycles with barcoding primers. The resulting libraries were submitted for Illumina SR50 sequencing. Average reads in each of the three replicates corresponding to each shRNA in each fraction were used to determine enrichment.

Retrovirus production

The Plat-E cell line was used to prepare ecotropic retroviral particles for T-cell transduction using Mirus (Mirus Bio) or jetPRIME (Polyplus) transfection reagents (Morita et al. 2000).

Electroporation of sgRNA amplicons

Naïve $Cas9^{Tg}$ CD8$^+$ T cells were sorted and immediately electroporated with 4D-Nucleofector (Lonza) using 0.5 μg of sgRNA amplicon obtained by PCR amplification from retroviral sgRNA vector. After electroporation, cells were activated for 48 h, transduced with the indicated retroviral sgRNAs (as described above), and cultured in TCM supplemented with IL-2 (as described above) for another 48–72 h before analysis.

HDAC and LSD1 inhibitor treatment

TSA (HDAC inhibitor; Sigma), GSK2879552 (LSD1 inhibitor; Cayman Chemical), or DMSO was added to T cells after 4 d of culture and analyzed either 24 or 48 h later by flow cytometry.

Bisulfite PCR sequencing

DNA was isolated from T cells using either phenol–chloroform extraction or Qiagen DNEasy kit. Purified genomic DNA was subjected to bisulfite treatment with the Qiagen EpiTect bisulfite kit and PCR-amplified with KAPA Taq DNA polymerase (Kapa Biosystems). The PCR amplicon coordinates shown are relative to the mm9 genome. Amplicons were TA-cloned using pGEM-T Easy Vector systems (Promega) and transformed into Stbl3 bacteria for subsequent plasmid extraction with Qiagen Spin Miniprep kit followed by sequencing. Analysis of Sanger-sequenced clones was performed with QUMA (Kumaki et al. 2008).

Whole-genome DNA methylation sequencing

DNA was isolated from T cells using phenol–chloroform extraction. For each sample, 100 ng of input DNA was sheared with a Covaris LE220 instrument aiming for 450-bp fragments. Sheared DNA was prepared with an EM-Seq kit (New England Biolabs) following the manufacturer's instructions. Briefly, the treatment included a TET2-mediated oxidation of 5mCs to 5hmCs. This was followed by APOBEC cytosine deaminase treatment, resulting in conversion of unmethylated cytosines to uracil and their appearance as thymidines in the final sequencing data. Methylated and hydroxymethylated cytosines were indistinguishable from each other and were present as cytosines in the sequencing data. Unmethylated (λ) and fully CpG methylated (pUC19) DNA were spiked into each sample and used as internal control for conversion efficiencies. The final sequencing libraries were amplified with four cycles of PCR, and libraries were sequenced on Illumina HiSeqX targeting 30× coverage per sample. After sequencing, the FASTQ files were processed as follows: Adapter sequences (Illumina TruSeq, "AGATCGAAGAGAC") were hard-clipped from raw paired-end FASTQs using Trim Galore version 0.4.4 (https://github.com/FelixKrueger/TrimGalore). The resulting reads were then aligned with default parameters for bwa-meth (https://github.com/brentp/bwa-meth) to a reference genome comprised of concatenated mm10, pUC19, and λ sequences, with the exception of inclusion of the "bwa mem -Y" flag and flagging of quality control-failed reads (0×200 flag) when alignment length was <0.2× read length. Aligned BAMs were split into separate mm10, pUC19, or BAMs for downstream analysis. These split BAMs were duplicate-marked using Picard version 2.8.0 (http://broadinstitute.github.io/picard). Methylation genotyping was performed on each using MethylDackel version 0.1.13 (https://github.com/dpryan79/MethylDackel). Methylation sequencing was deposited in the Gene Expression Omnibus (GEO) database under accession number GSE126472.

ATAC-seq

Samples were prepared as described (Buenrostro et al. 2013). Briefly, WT or $Cas9^{Tg}$ T cells were activated and transduced with retroviral shRNA constructs or electroporated with sgRNA, respectively. After five additional days of culture, 50,000 cells were sorted based on CD8 and CD4 expression. Cells were washed, nuclei were isolated, and libraries were submitted to the New York University Langone Genome Technology Center for sequencing. Fastq files were obtained from 50-bp paired-end reads and analyzed by aligning reads to the mm9 genome with Bowtie2 using default settings on the Galaxy platform (Afgan et al. 2018). The resulting BAM files were filtered for PCR duplicates (using sambamba), mitochondrial DNA alignments, MAPQ <30 alignment quality, ENCODE blacklisting, and imperfect paired-end reads. Filtered BAM files were down-sampled when possible to ∼13 million filter-aligned reads, the third lowest complexity-filtered BAM file size, before visualization on Integrative Genomics Viewer by generating bigWig files with bamCoverage on default settings on the Galaxy platform (Robinson et al. 2011). ATAC-seq data were deposited in the GEO database under accession number GSE126472.

RNA-seq

T-cell samples were cultured in the same way as ATAC-seq (above). RNA was isolated from cells sorted into TRIzol (Invitrogen) followed by treatment with DNase I and cleanup with RNeasy MinElute kit (Qiagen). T-cell RNA libraries were prepared with Nugen Ovation Ultralow library system V2 by HudsonAlpha. Sequencing was performed with Illumina HiSeq 2500 version 4. FASTQ files were processed through kallisto using an mm10 kallisto index from an ENSEMBL GTF annotation file. Count estimates from kallisto were extracted and aggregated to gene-level counts by summation. Principal component analysis (PCA) was conducted using prcomp on a CPM matrix filtered for genes that had >5 CPM in at least two samples. Differential expression analysis was conducted on the entire count matrix using voom and limma, with an additive model for the main perturbational effect (i.e., shDnmt1) and the CD4$^+$ effect to control for variability seen in the PCA. Differential expression genes were considered with an FDR of <0.05 (Benjamini-Hochberg-corrected P-value) and an absolute log$_2$(fold change) of >1. RNA-seq data were deposited into the GEO database under accession number GSE126472.

Combined genome-wide analysis of ATAC-seq and RNA-seq

Differentially expressed genes from RNA-seq described above were used for analysis as follows: ATAC-seq peaks and reads within those peaks were annotated to the nearest gene TSS within 30 kb using the Bedtools closest command. Unannotated peaks were discarded. Reads within peaks were then aggregated down to gene level by summation, and differential expression analysis was annotated as with RNA-seq and plotted by log$_{10}$ fold change.

Western blotting

For Western blotting, samples were transferred from polyacrylamide gel to nitrocellulose membrane by wet transfer for 4 h on ice at 60 V. Blots were blocked in PBS blocking buffer (Licor) and then stained overnight with primary antibody at 4°C. The next day, membranes were washed three times in PBST (PBS and 0.1% Tween-20), stained with fluorescently conjugated secondary antibodies (Licor) at 1:10,000 dilution in PBS blocking

buffer for 1 h at room temperature, and then imaged in the 680- and 800-nm channels.

Flag tag coimmunoprecipitation and mass spectrometry

Transfected Plat-E cells were cultured for 48–72 h, detached from 10-cm dishes by pipetting, and subsequently washed in PBS. Nuclei were isolated by resuspending cell pellets in 500 μL of IPMS buffer (0.5% NP-40, 100 mM NaCl, Tris-HCl at pH 8.0, 1× protease inhibitor [Sigma]) and rotating for 30 min at 4°C. Samples were homogenized by passing through a 23.5-gauge needle 10–15 times. Cellular debris was spun down, and the supernatant was precleared with protein A beads (Dyna beads) for 1 h prior to incubation with 10 μg of Flag antibody overnight at 4°C with rotation. For samples used to assess for histone binding (Supplemental Fig. S8C), 0.625 μg of human recombinant H3.1–H4 tetramer (New England Biolabs) was spiked in. The next day, protein-bound beads were washed five times in IPMS buffer, and bound proteins were eluted by boiling samples in loading buffer. Samples were either submitted to New York University School of Medicine Proteomics Laboratory for mass spectrometry or used for Western blot. The mass spectrometry proteomics data were deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD012669 (Perez-Riverol et al. 2019).

ChIP-qPCR

T cells were transduced and cultured for 5 d. Next, 100,000 CD8+CD4− shRenilla.713-treated or CD8+CD4+ shChaf1a.3118-treated T cells were isolated by cell sorting and processed for ultralow input micrococcal nuclease-based native ChIP (ULI-NChIP) (Brind'Amour et al. 2015). For immunoprecipitation, 3 μg of antibody was used per reaction. Immunoprecipitated and input DNA were used for qPCR with SYBR Green 2× master mix (Roche).

Cell sorting and flow cytometry

Samples were FACS-sorted on a FACSAria (BD Biosciences). Flow cytometry was performed on an LSRII (BD Biosciences), and analysis was performed using Flowjo (Treestar).

Acknowledgments

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