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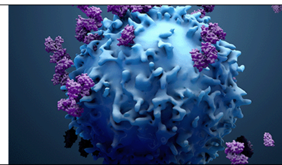
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Epigenetic Silencing of *Cd8* Genes by ThPOK-Mediated Deacetylation during CD4 T Cell Differentiation

Jinxiu Rui, Haifeng Liu, Xiaoyan Zhu, Yu Cui, and Xiaolong Liu

Intrathymic CD4/CD8 differentiation is a process that establishes the mutually exclusive expression profiles of the CD4 and CD8 T cell lineage. The RUNX3-mediated silencing of CD4 in CD8 lineage cells has been well documented; however, it is unclear how CD8 is silenced during CD4 lineage differentiation. In this study, we report that, by directly binding the CD8 locus, ThPOK works as a negative regulator that mediates the deacetylation of *Cd8* genes and repositions the CD8 alleles close to heterochromatin during the development of the CD4 lineage. The ectopic expression of ThPOK resulted in increased recruitment of histone deacetylases at *Cd8* loci; the enhanced deacetylation of *Cd8* genes eventually led to impaired *Cd8* transcription. In the absence of ThPOK, the enhanced acetylation and transcription of *Cd8* genes were observed. The results of these studies showed that *Cd8* loci are the direct targets of ThPOK, and, more importantly, they provide new insights into CD8 silencing during CD4 lineage commitment. ***The Journal of Immunology*, 2012, 189: 1380–1390.**

During the development of αβ T cells, the alternate commitment to CD4 and CD8 lineages is important for the generation of distinct helper and killer T cells. This process involves establishing perfect matching between TCR specificity toward MHC class I or MHC class II and the mutually exclusive expression of CD8 and CD4 surface markers, respectively (1, 2). Recently, extensive efforts have been devoted to elucidating the mechanism by which CD4 and CD8 expression is regulated. Accumulating evidence indicates that CD4 and CD8 are primarily regulated at the transcriptional level through a combination of enhancers and/or silencer elements. The regulation of CD4 expression occurs through the association of a lineage-specific silencer with ThPOK or RUNX proteins in the CD4 or CD8 lineage (3–5). In contrast, the regulation of CD8, which usually involves CD8α and CD8β heterodimers in thymic-derived T cells, is more complicated owing to multiple stage-specific enhancers, $E8_I$–$E8_V$, located between the *Cd8a* and *Cd8b* promoters (6, 7). However, no *Cd8* silencer has yet been identified, and the negative regulation of CD8 expression is largely unexplored. The negative regulation of *Cd8* enhancers has been proposed as a mechanism for CD8 silencing. Several NFs, including IKAROS, RUNX1, RUNX3, and STAT5, have been implicated in the maintenance of CD8 expression through physical association with *Cd8* enhancers (4, 8, 9). MAZR is a negative regulator that directly binds to *Cd8* enhancers in double-negative (DN) thymocytes (10). However, no factor has yet been reported to be involved in CD8 silencing during the differentiation of CD4 single-positive (SP) cells.

ThPOK (also known as cKrox or Zbtb7b) is an important regulator of the CD4/CD8 lineage commitment. The functional deficiency or overexpression of ThPOK perturbs normal lineage differentiation and causes double-positive (DP) thymocytes to develop exclusively into the CD8 or CD4 lineage. ThPOK is a CD4 T cell commitment factor (11) that can be silenced through RUNX3 or MAZR in the CD8 lineage (12, 13). Additionally, ThPOK occupies both silencers in the CD4 lineage to confer protection from RUNX proteins (4). Interestingly, thymocytes that are deficient in both ThPOK and RUNX3 are committed by default to the CD4 lineage (14), suggesting that ThPOK antagonizes RUNX3-mediated CD8 lineage choices in the process of CD4/CD8 lineage commitment. Indeed, the overexpression of ThPOK represses cytotoxic gene expression in CD4 (15) or mature CD8 T cells (16). However, it is still unknown whether ThPOK is responsible for CD8 silencing in the commitment of the CD4 lineage.

Given the upregulation of ThPOK and the silencing of CD8 in the development of CD4 SP thymocytes (17), we sought to determine the possible involvement of ThPOK in CD8 silencing during this process using ThPOK-transgenic and ThPOK-deficient mice. In this study, we demonstrated that ThPOK stably represses CD8 expression through the deacetylation of *Cd8* loci in CD4 lineage commitment.

Materials and Methods

Mice

AND OT-1 TCR-transgenic mice were obtained from The Jackson Laboratory. The ThPOK-transgenic (C8 line) and $ThPOK^{-/-}$ mice were gifts from Dr. R. Bosselut (National Institutes of Health, Bethesda, MD). All mice were maintained in a specific pathogen-free facility and analyzed at 4–8 wk of age. The Institutional Animal Use Committee of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, approved all animal experiments.

Abs and reagents

The following Abs were obtained from BD Pharmingen and used for surface staining: anti-CD4 (GK1.5), anti-CD8α (53-6.7), and anti-CD8β (53-5.8).

State Key Laboratory of Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

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Address correspondence and reprint requests to Prof. Xiaolong Liu, State Key Laboratory of Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yueyang Road, Shanghai 200031, China. E-mail address: liux@sibs.ac.cn

The online version of this article contains supplemental material.

Abbreviations used in this article: ChIP, chromatin immunoprecipitation; DN, double-negative; DP, double-positive; FISH, fluorescence in situ hybridization; HDAC, histone deacetylase; IP, immunoprecipitation; Q-PCR, quantitative real-time PCR; RTOC, reaggregate thymic organ culture; SP, single-positive; TSA, trichostatin A; WT, wild-type.

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A mouse Ab directed against a GST fusion protein containing 152–351 aas of mouse ThPOK was derived using conventional methods. Mouse anti-HDAC4 (ab-1437) was obtained from Abcam. Pronase (P8811), anti-Flag (M2) mAb (F3165), and the affinity gel (A2220) were obtained from Sigma-Aldrich. The anti–hemagglutinin-probe (Y-11; sc-805), mouse anti-HDAC5 (sc-133225), goat anti-HDAC4 (sc-5245), and mouse and rabbit IgG were purchased from Santa Cruz Biotechnology. Anti-Ace-H3/H4, protein A/G-agarose/ssDNA, and trichostatin A (TSA; 19-138) were obtained from Millipore.

Cell lines and transfection

HEK 293T cells, Plat-E cells, and RLM-11 cells (a gift from Dr. Ichiro Taniuchi, RIKEN Research Center for Allergy and Immunology, Yokohama, Japan) were maintained in DMEM. HEK 293T and Plat-E cells were transfected using calcium phosphate precipitation. RLM-11 cells were transfected using electroporation (265 V, 975 μF, 4 mm cuvette; Gene Pulser Xcell; Bio-Rad Laboratories).

Cell preparation, staining, and purification

The single-cell thymocyte suspension was prepared and surface stained as previously described (18). Thymocyte subsets were sorted using a FACSAria II cell sorter (BD Biosciences), and the cell purity was >90%. Peripheral CD8 T cells were obtained from the lymph nodes of wild-type (WT; C57BL/6) mice and purified using a Dynal mouse CD8 cell negative isolation kit (Invitrogen, Carlsbad, CA).

RT-PCR and quantitative real-time PCR

RNA was extracted and quantified as previously described (18) and reverse-transcribed using the SuperScript III First-Strand kit (Invitrogen). The mRNA level of *Cd8* was assessed according to the relative abundance of *Hprt* using quantitative real-time PCR (Q-PCR; Rotor gene 6000; Corbett Life Sciences) with SYBR Green Master Mix (Toyobo). The following primers were used for Q-PCR: *Cd8a* (5′-TCATCCCACAACAAGATAA-CG-3′, 5′-TCTGAAGGACTGGCACGAC-3′), *Cd8b* (5′-GATGGTCTT-TGGGACAGGG-3′, 5′-ATGCCAGCAGAAGCAGGAT-3′), and *Hprt* (5′-CCTGCTGGATTACATTAAAGCACT-3′, 5′-TTCAACACTTCGAGAG-GTCCT-3′).

Immunoprecipitation and immunoblotting

At 48 h after transfection, HEK 293 T cells were collected, washed, and lysed in lysis buffer (10 mM Tris-HCl [pH 7.4], 300 mM NaCl, 1 mM EDTA, 1% [v/v] Triton X-100, and 10% [v/v] glycerol) containing protease inhibitors (Roche) on ice for 30 min. The cell debris was removed, and Flag M2 affinity gel was added to the lysate for Flag precipitation overnight at 4°C with gentle rotation. The immune complexes were pelleted, washed, and eluted in SDS loading buffer. For in vivo immunoprecipitation (IP), 50×10^6 total thymocytes were lysed in lysis buffer containing 150 mM NaCl. The samples were analyzed using 12% SDS-PAGE and immunoblotting.

Luciferase reporter assay

The *Cd8a* promoter (391 bp), *Cd8b* promoter (−1150 to +50), $E8_I$ (8 kb), $E8_{II}$ (3.9 kb, without the 600-bp 5′ flanking sequences), $E8_{III}$ (4 kb), $E8_{IV}$ (4.5 kb), and $E8_V$ (3 kb) were obtained from the CD8 BAC clone (RP23-322E20; Invitrogen). The *Cd8* promoter was cloned into PGL3 basic, and $E8_I$–$E8_V$ were inserted upstream of the *Cd8* promoter to generate the E8–P8-luciferase reporter construct. RLM-11 cell suspensions were harvested at 18–24 h after transfection, and luciferase activity was determined using a Dual-Luciferase reporter kit (Promega).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays (Upstate Biotechnology and Diagenode) were performed according to the manufacturers' instructions with modifications. Purified subsets of thymocytes or retroviral-transduced CD8 T cells were crosslinked for 30 min on ice using 1% (v/v) formaldehyde and lysed for 10 min on ice. The lysates were sonicated in a 4°C water bath (Bioruptor UCD-200) to obtain DNA fragments of an average length of 500 bp. The fragmented lysates were subjected to IP with the indicated Abs. The recovered DNA was detected using Q-PCR with specific primers spanning the *Cd*8 loci. The following primers were used for ChIP analysis: P8b (5′-TCTTCTTCTGTGGTGGATG-3′, 5′-TCTTCTT-CTGTGGTGGATG-3′), $E8_{IV}$-1 (5′-CACCAACCAAGAAGACTACC-3′, 5′-CTAGAGAAATAGGCTACAGAGC-3′), $E8_{III}$-1 (5′-GTCTGGTCTAC-ACAGTGAGGA-3′, 5′-GCTGATCTGAGGGACTATTG-3′), $E8_{III}$-2 (5′-GCTGATCTGAGGGACTATTG-3′, 5′-GTGTTTGTTCTAGCCGTTGGAG-3′), $E8_{II}$-1–7 (5′-GTAATAAGATAAGGGGGAAAGACC-3′, 5′-GTAAT-AAGATAAGGGGGAAAGACC-3′), $E8_I$-1 (5′-TTTTCATTGTTGGGA-AGCAC-3′, 5′-CAAAGCAGGTTGAGGCAC-3′), $E8_I$-2, 3 (5′-AAGTTC-ATTGTGCCCTAGACTCCC-3′, 5′-AGGTGTGCAGCTGGGAGCTT-3′), $E8_I$-4 (5′-AAAGGAAGGAAGAAGAACG-3′, 5′-TCAGAAGCCAAAG-AATCAC-3′), $E8_I$-5 (5′-TAAAGGTGTGCTGGCTGT-3′, 5′-GGTGGA-AATCTGCTGTCAT-3′), $E8_I$-6 (5′-TTTCCCTTGGCTTATTTGCT-3′, 5′-ATACCACCTCCCCACACACC-3′), $E8_I$-7 (5′-GATTGCTTTCTGGGA-CACT-3′, 5′-GGATTTTATTACAAGGGGC-3′), $E8_V$-1 (5′-AAGGCTGT-TGACCTAAGTTC-3′, 5′-AATACCCAAATGCTGACTCT-3′), P8a (5′-ACCGTTGACCCGCTTTCTGT-3′, 5′-CCAAGTTCGGCGTCCATTTT-3′), S4 (5′-GTAGGCACCCGAGGCAAAG-3′, 5′-GGAGGGCACAGTTCCA-GCA-3′), and Actb (5′-GTCTTTCTTCTGCCGTTCT-3′, 5′-CACTTATC-ACCAGCCTCAT-3′).

Three-dimensional DNA fluorescence in situ hybridization and confocal analysis

The sorted cells were washed in PBS and fixed in 0.5% glutaraldehyde on poly-L-lysine–coated slides for two-color three-dimensional DNA fluorescence in situ hybridization (FISH) analysis as previously described (19). Briefly, chromosomal DNA was denatured by a 3-min NaOH treatment (pH, 12–13.2). The CD8 BAC clone RP23-139M18 (a gift from Dr. Jane Skok) was labeled with DIG-11-dUTP using DIG-Nick translation mix (Roche) to create a DNA probe for the *Cd8* gene. The probe used for the detection of pericentromeric heterochromatin was constructed as previously described (20) and composed of eight tandem copies of the main γ-satellite repeat sequence and was directly labeled by with Cy3-dUTP (GE Healthcare) according to the manufacturer's advice. The cells were analyzed with confocal microscopy on a Leica SP2 acoustica optical beam splitter system. Optical sections separated by 0.4 μm were collected, and only cells with signals from both alleles were analyzed. The distances between signals were measured on individual Z focal planes. Alleles displaying overlapping or immediately juxtaposed γ-satellite signal were defined as pericentromerically localized if the γ-satellite signal was overlapping or immediately juxtaposed. A minimum of 100 cells were analyzed for pericentromeric association.

Retroviral gene transfer into CD8 T cells or developing thymocytes and reaggregate thymic organ cultures

Retrovirus infection was performed as previously described (21). Briefly, Plat-E cells were transfected with the indicated ThPOK derivatives on pMCs-IRES-GFP, and supernatant containing retrovirus was collected at 48–72 h posttranfection to transduce purified CD8 T cells (after 1 d activation with plastic-coated anti-CD3 [1 μg/ml] and anti-CD28 [2 μg/ml]) or DN thymocytes prepared from day 15.5 C57BL/6 embryos by spin infection at $1000 \times g$ for 90 min at 30°C in a 24-well plate. After transfection, an aliquot of DN cells was cultured overnight and subsequently assessed by FACS analysis to determine the efficiency of transduction. The remaining cells were reaggregated with deoxyguanosine-prepared fetal thymus for 7–10 d before FACS analysis.

Statistical analysis

A 4-fold table χ^2 analysis of the FISH assay was used to calculate the *p* values.

Results

ThPOK associates with multiple sites at the Cd8 *locus*

We first analyzed the sequence of a 20-kb DNA fragment at the *Cd8* locus containing *Cd8* promoters (*Cd8a* and *Cd8b*) and *Cd8* enhancers ($E8_I$–$E8_V$) using Vector NTI software for the consensus ThPOK binding sequence GGGAGGG (22, 23). There were 20 GGGAGGG sequences in total at the studied loci (Fig. 1A). Many potential guanine-rich ThPOK binding sequences that are less conserved were also present (data not shown).

We performed anti-ThPOK ChIP assays with polyclonal mouse serum in total thymocytes from AND TCR-transgenic mice as a pilot experiment. We observed that ThPOK specifically binds to multiple sites at the *Cd8* loci in total thymocytes (Fig. 1B), encompassing all enhancers and the *Cd8a* promoter (P8a). No enrichment at the *Cd8b* promoter (P8b) was detected. We also performed a ChIP assay to further verify the association of ThPOK with *Cd8* loci in sorted DP

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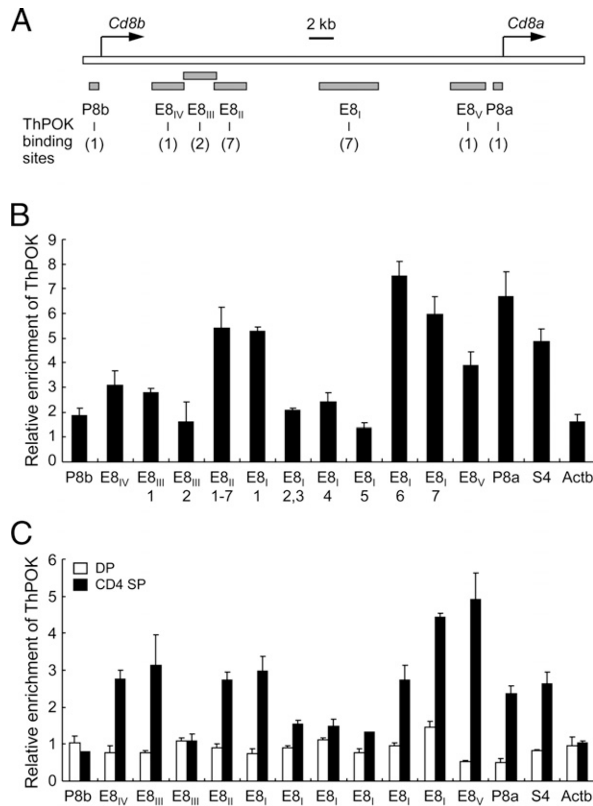

FIGURE 1. ThPOK associates with multiple sites in the *Cd8* loci. (**A**) Schematic map of the *Cd8b–Cd8a* loci. The location and transcriptional orientation of the *Cd8a* and *Cd8b* genes are shown. The previously reported enhancer and promoter regions are indicated. The number of consensus ThPOK-binding sites for each element is noted in the parentheses. (**B** and **C**) ThPOK binds to the *Cd8* loci in vivo. Recruitment of ThPOK to the *Cd8* loci was assessed by ChIP assay using anti-ThPOK mouse serum, and preimmune mouse serum was included as an isotype control. The presence of *Cd8* promoter/enhancer sequences in the Ab-bound chromatin segments was analyzed using Q-PCR. The data obtained were normalized on the basis of the corresponding isotype control, and the means ± SD from three independent experiments were plotted. Relative ThPOK occupancy is shown at *Cd8* elements in total thymocytes from AND TCR-transgenic mice (B) or DP and CD4 SP cells from WT mice (C) at 20 sites spanning the *Cd8* loci. S4 (*Cd4* silencer) and Actb (β-actin) were positive and negative controls, respectively.

and CD4 SP subsets from WT mice. ThPOK was recruited to multiple sites in the *Cd8* loci of CD4 SP cells (Fig. 1C). This enrichment was not detected in DP subsets where ThPOK is not expressed, indicating the specificity of the ThPOK Ab in the ChIP assay. Because ThPOK recognition sites have not been definitively characterized, we obtained comprehensive information concerning ThPOK binding to *Cd8* loci by mapping every 500 bp of ThPOK binding in the *Cd8* loci. The results showed that, in addition to the consensus binding sites, ThPOK was associated with less conserved sequences as well (data not shown). Consistent with previous reports (4), ThPOK enrichment at the *Cd4* silencer (S4) was also detected (Fig. 1B, 1C). Taken together, these results suggest that ThPOK can specifically associate with *Cd8* loci, including all stage-specific *Cd8* enhancers and the *Cd8a* promoter.

ThPOK functions as a negative regulator of the Cd8 *promoter and enhancers*

Given the comprehensive association of ThPOK with *Cd8* enhancers and the *Cd8a* promoter, we wondered whether ThPOK represses the transcriptional activity of these elements. The results of the luciferase reporter assay indicated that ThPOK effectively repressed the *Cd8a* promoter in a dose-dependent manner, whereas the transcription activity of *Cd8b*, which was initially low in our assay, was not affected (Fig. 2A). These results were consistent with our earlier observation that no detectable ThPOK enrichment at the *Cd8b* promoter was observed (Fig. 1B, 1C).

Because ThPOK expression did not influence the *Cd8b* promoter in the reporter assay, *Cd8* enhancers were individually inserted upstream of the *Cd8b* promoter to examine the influence of ThPOK. As shown in Fig. 2B, ThPOK efficiently suppressed four of the five enhancers. ThPOK did not inhibit $E8_{II}$, which marginally enhanced the transcriptional activity of the *Cd8a* and *Cd8b* promoters in the reporter assay. Although an obvious enrichment of ThPOK was detected in vivo (Fig. 1B, 1C), the 600-bp region in the 5′ flanking sequence of $E8_{II}$ that we failed to obtain might explain this result. Taken together, our results indicate that ThPOK is a negative regulator of the *Cd8a* promoter and *Cd8* enhancers.

We further confirmed the repression by inserting $E8_I$–$E8_V$ upstream of the *Cd8a* promoter. As predicted, the combination of the *Cd8a* promoter and *Cd8* enhancers increased ThPOK repression (Fig. 2C). The ThPOK-mediated repression in the presence of *Cd8* enhancers was calculated by normalizing *Cd8* promoters from Fig. 2B and 2C. The results show that ThPOK efficiently represses the *Cd8* enhancers (Fig. 2D). Therefore, ThPOK functions as a negative regulator of *Cd8 cis* elements.

Class II HDAC-induced deacetylation is involved in CD8 repression

ThPOK belongs to the BTB/POZ family of transcriptional regulators. For several members of this family, transcriptional repression is achieved through BTB domain-mediated interactions with various corepressors and HDACs (24–26). To investigate the possible involvement of HDACs in the ThPOK-mediated repression of *Cd8 cis* elements, we used TSA, an inhibitor of class I and class II HDACs, in a reporter assay. Interestingly, ThPOK failed to repress *Cd8 cis* elements in the presence of TSA. Moreover, an enhanced CD8 level was observed in $CD4^+CD8^{low}$ but not DP thymocytes from WT mice after overnight culture in the presence of TSA (data not shown), suggesting a possible role for HDACs in ThPOK-mediated CD8 repression. The primary screen for ThPOK-interacting HDACs showed that HDACs 3, 4, 5, and 10 were associated with ThPOK and belong to class II HDACs, except HDAC3 (Fig. 3A). In the reporter assay, CD8 was repressed through HDACs 4, 5, and 10 alone or in combination with ThPOK to induce increased repression (Fig. 3B, *top*). The repression was also sensitive to TSA treatment (Fig. 3B, *bottom*). Notably, *Cd8* was not repressed by HDAC3 alone or in combination with ThPOK, probably because it is not a class II HDAC. Similar results were obtained when *Cd8* enhancers were used as substrates in our reporter assays (data not shown), suggesting that class II HDACs are involved in CD8 repression through ThPOK in vitro.

We then evaluated the involvement of class II HDACs in the regulation of CD8 in vivo. HDAC4/5 and ThPOK coprecipitated in total thymocytes (Fig. 3C). HDAC10 was not included because of the unavailability of an appropriate Ab. HDAC4 and HDAC5 are structurally similar and can associate with each other (27) (Fig. 3C); therefore, we focused on HDAC4 rather than duplicating all of the experiments with HDAC5. The anti-HDAC4 ChIP assay was performed in DP and CD4 SP cells from WT mice to determine the association of HDAC4 with the studied *Cd8* regions. HDAC4 occupied the same sites at *Cd8* loci as ThPOK in CD4 SP cells (Fig. 3D). However, HDAC4 did not occupy these sites in the DP cells, indicating the deposition of HDAC4 through ThPOK at *Cd8* loci. The co-occupation of HDAC4 with ThPOK at *Cd8* loci

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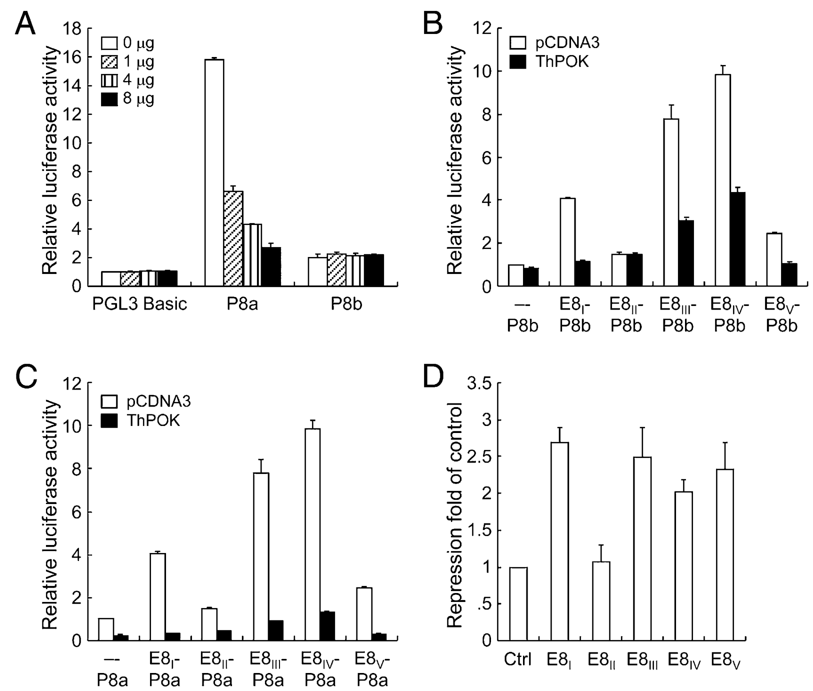

FIGURE 2. ThPOK functions as a negative regulator of the *Cd8* promoter and enhancers. (**A**–**C**) Luciferase activity of lysates of RLM-11 cells electroporated with a *Cd8* promoter/luciferase reporter together with the indicated amount of ThPOK expression plasmid (A), $E8_I$–$E8_V$ fragments inserted upstream of *Cd8b* (B), or a *Cd8a* promoter-luciferase reporter (C). In (A)–(C), the amount of DNA was kept constant using pCDNA3. Luciferase activity was assayed at 24 h after electroporation. The data shown are from three independent experiments with similar results (means ± SD). (**D**) The ThPOK-mediated repression in the presence of *Cd8* enhancers was calculated by normalizing the *Cd8b* (B) and *Cd8a* promoters (C). The data shown are the average of four independent experiments (means ± SD).

would presumably influence the acetylation of histones and the accessibility of DNA in *Cd8* loci. Therefore, an anti–Ace-H3/H4 ChIP assay was employed in DP (in which most of the *Cd8* enhancers are active), $CD4^+CD8^{low}$ (intermediate progenitors of both the CD4 and the CD8 lineages), and CD4 SP cells (in which most of the *Cd8* enhancers are inactive) from WT mice. Interestingly, histones H3 and H4 that were located at many of the potential ThPOK-binding sites changed from a highly acetylated to an extremely low or nonacetylated status during the DP–CD4 SP transition (Fig. 3E).

The recruitment of class II HDACs is a prerequisite for ThPOK-mediated CD8 repression

Based on the results obtained above, we proposed a possible link between ThPOK, HDACs, and *Cd8* genes in vivo. We first screened for the domain responsible domain for ThPOK and HDACs association and observed that the ThPOK BTB domain was indispensable for class II HDAC recruitment, ThPOK dimerization (BTB/POZ transcription factors usually function as dimers) (28, 29), and proper ThPOK functioning (data not shown). These observations agree with the undisturbed CD4/CD8 lineage in previously reported ThPOKΔBTB-transgenic mice (17). To further determine the roles of HDACs in ThPOK function, minimal mutations in conserved amino acids among BTB domains of different POK factors were introduced (29, 30) and two mutants, designated ThPOKM1 (L21S) and ThPOKM2 (QR27/28AL), did not interact with HDAC4/5 (Fig. 4A) and failed to repress CD8 expression when overexpressed in CD8 T cells through retroviral transfection (Fig. 4B). Notably, ThPOKM1/M2 self-associated and associated with ThPOK (Fig. 4C) and could bind the same *Cd8* elements as did ThPOK when introduced into CD8 T cells as shown for representative sites on $E8_I$, $E8_V$, and P8a (Fig. 4D, *left*). However, ThPOKM1 and ThPOKM2 failed to deposit HDAC4 to the studied regions (Fig. 4D, *right*).

To investigate the in vivo role of class II HDACs in ThPOK-mediated regulation of CD8 expression, day 15.5 mouse fetal thymocytes were transduced with ThPOK or ThPOKM1/M2 retroviral supernatant followed by reaggregate thymic organ cultures (RTOC). The GFP levels in the transduced thymocytes were similar (Supplemental Fig. 1A). As described in previous reports, the cells from RTOC underwent normal development with a slight increase in the relative proportion of DN and CD8 SP cells, which could be due to the targeting of cycling DN cells (21, 31, 32). Upon reconstitution, the surface CD8 level on the $CD4^+CD8^{low}$ subset was substantially perturbed in ThPOK cultures but remained undisturbed in ThPOKM1/M2 or the control RTOC (Fig. 4E, 4F). Unlike ThPOK-transgenic (C8) mice (17), a large percentage of thymocytes were blocked at the DN stage in ThPOK-transduced RTOC, although the lineage reversion from CD8 to CD4 was remarkable compared with the GFP^- control. In contrast, both ThPOKM1 and M2 failed to promote lineage reversion from CD8 to CD4 but generated more CD8 SP cells and less CD4 SP cells compared with their GFP^- controls (Fig. 4E), suggesting that the introduced mutants competed with the endogenous ThPOK in the CD4/CD8 lineage decision. We further analyzed the ratio of transduced versus nontransduced mature (HSA^{low}) CD4 SP and CD8 SP cells. The ratio was much lower in CD4 SP cells and substantially higher in CD8 SP cells from both ThPOKM1/M2 RTOC (Supplemental Fig. 1B, 1C). As the results of the reporter assay demonstrated, both mutants could compete with ThPOK and release CD8 *cis* elements from ThPOK-mediated suppression (data not shown). Taken together, the recruitment of class II HDACs is a prerequisite for ThPOK-mediated CD8 repression and CD4/CD8 lineage reversion.

CD8 repression by ThPOK depends on its DNA binding ability

The extensive association of ThPOK with CD8 regulatory regions via consensus and less conserved sequences raised questions concerning the significance of the consensus sites and whether the role of ThPOK in CD8 repression was direct. To address these questions, we introduced mutations into the consensus binding sites (GGGAGGG to GTAAGGG) (33) in the promoter, enhancer, or both elements of the $E8_v$–P8a reporter as well as in the enhancer of the $E8_{III}$–P8a reporter. The results showed that in the presence of ThPOK, the transcription activity driven by the mutated reporters was restored, although not completely reversed, after both consensus binding sites were mutated in the $E8_v$–P8a reporter or in the enhancer of the $E8_{III}$–P8a reporter (Fig. 5A),

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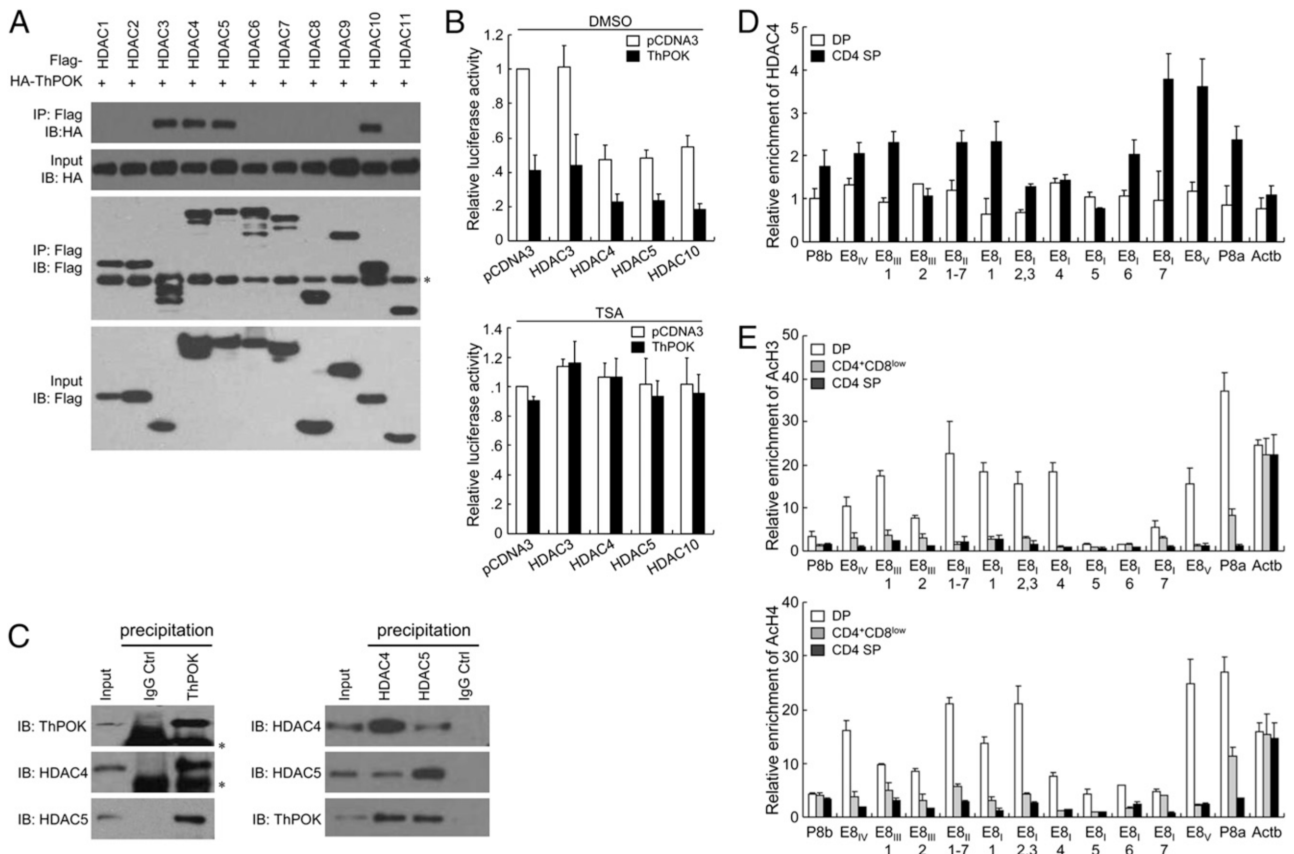

FIGURE 3. Class II HDACs are involved in CD8 suppression. (**A**) Interaction of ThPOK with class II HDACs. Coimmunoprecipitation was performed to screen for HDACs that interact with ThPOK. The results are representative of three independent experiments. *, Cross-reacting IgG; IB, immunoblot. (**B**) Effects of ThPOK-interacting HDACs on *Cd8* loci in the absence or presence of ThPOK. Luciferase activity in the lysates of RLM-11 cells electroporated with the indicated expression plasmids together with the *Cd8a*-luciferase reporter. Transfected cell suspensions were split in half 6 h after transfection. One half of each suspension was supplemented with DMSO vehicle (*top*), whereas the other half was supplemented with 200 nM TSA (*bottom*). The data shown are the average of three independent experiments (means ± SD). (**C**) In vivo IP assay with anti-ThPOK polyclonal mouse serum to precipitate HDAC4/5 (*left*) or anti-HDAC4/5 Ab to precipitate ThPOK and HDAC4/5 (*right*) in total thymocytes. The results are representative of three independent experiments. *, Nonspecific signal; IB, immunoblot. (**D**) The relative occupancy of HDAC4 at *Cd8* loci. Q-PCR analysis of recovered DNA from the anti-HDAC4 ChIP assay that was performed using indicated thymocytes from WT mice. Means ± SD from three independent experiments were plotted. (**E**) The relative abundance of hyperacetylated histone H3 (AcH3, *top*) and H4 (AcH4, *bottom*) at ThPOK-binding sites in sorted subsets from WT mice. Q-PCR analysis of recovered DNA from the indicated ChIP assay is shown. Assays were repeated three times with three independent samples (means ± SD).

suggesting a potential direct role for ThPOK in *Cd8* transcription; moreover, less conserved binding sites other than GGGAGGG could be responsible for ThPOK binding at *Cd8* elements.

To further address whether the role of ThPOK was direct, we alternatively examined whether the DNA binding ability was indispensable for proper ThPOK function. The ThPOK point mutation HD (1165A/G), referred to as ThPOKHD, is a well-documented derivative of ThPOK. The Arg to Gly substitution occurs within the second of four zinc finger domains in ThPOK and affects a residue predicted to interact directly with DNA (34, 35). We showed that ThPOKHD formed dimers and interacted with class II HDACs normally (Fig. 5B, 5C). However, when introduced into CD8 T cells, ThPOKHD could neither associate with *Cd8* elements nor bring HDACs as did ThPOK, as representatively demonstrated by several sites on $E8_I$, $E8_V$, and P8a (Fig. 5D). We also confirmed the dysfunction of ThPOKHD on CD8 expression in retroviral-transduced CD8 T cells (Fig. 5E).

Collectively, the results suggest that ThPOK plays a direct role in CD8 transcriptional repression, which requires its DNA binding ability. Moreover, these observations reveal that the HD form can neither bind nor bring HDACs to *Cd8* genes, which could partly explain the Th cell deficiency observed in HD mice.

Cd8 loci become less accessible in the presence of transgenic ThPOK during the differentiation of CD4 T cells

We thus examined ThPOK-mediated *Cd8* silencing in the differentiation of DP into CD4 SP cells using ThPOK-transgenic (C8) mice. As a result of ectopic expression, ThPOK effectively associated with *Cd8* loci early in the DP cells from C8 mice (Supplemental Fig. 2A). *Cd8a* and *Cd8b* transcription was repressed by ∼50% in both transgenic subsets (Supplemental Fig. 2B, 2C). However, no significant changes in CD8 expression were observed directly in fresh transgenic mice compared with WT subsets (Supplemental Fig. 2D), probably due to the population shift as a result of ThPOK being primarily a commitment factor for CD4 lineage. Therefore, we alternatively used markers as CD4, TCRβ, and CD69 to define the target cell population. Indeed, lower CD8 expression was observed in TCR-signaled thymocytes, including preselected DP and $CD4^+CD8^{low}$ cells from C8 mice (Supplemental Fig. 2E), which in turn suggests that ThPOK functions in differentiating TCR-signaled thymocytes.

We wondered whether the changes in CD8 levels would be more substantial in ThPOK and OT-1 TCR double-transgenic mice (referred to as C8/OT-1). The CD8 levels in both fresh DP and $CD4^+CD8^{low}$ subsets from C8/OT-1 mice were indeed substan-

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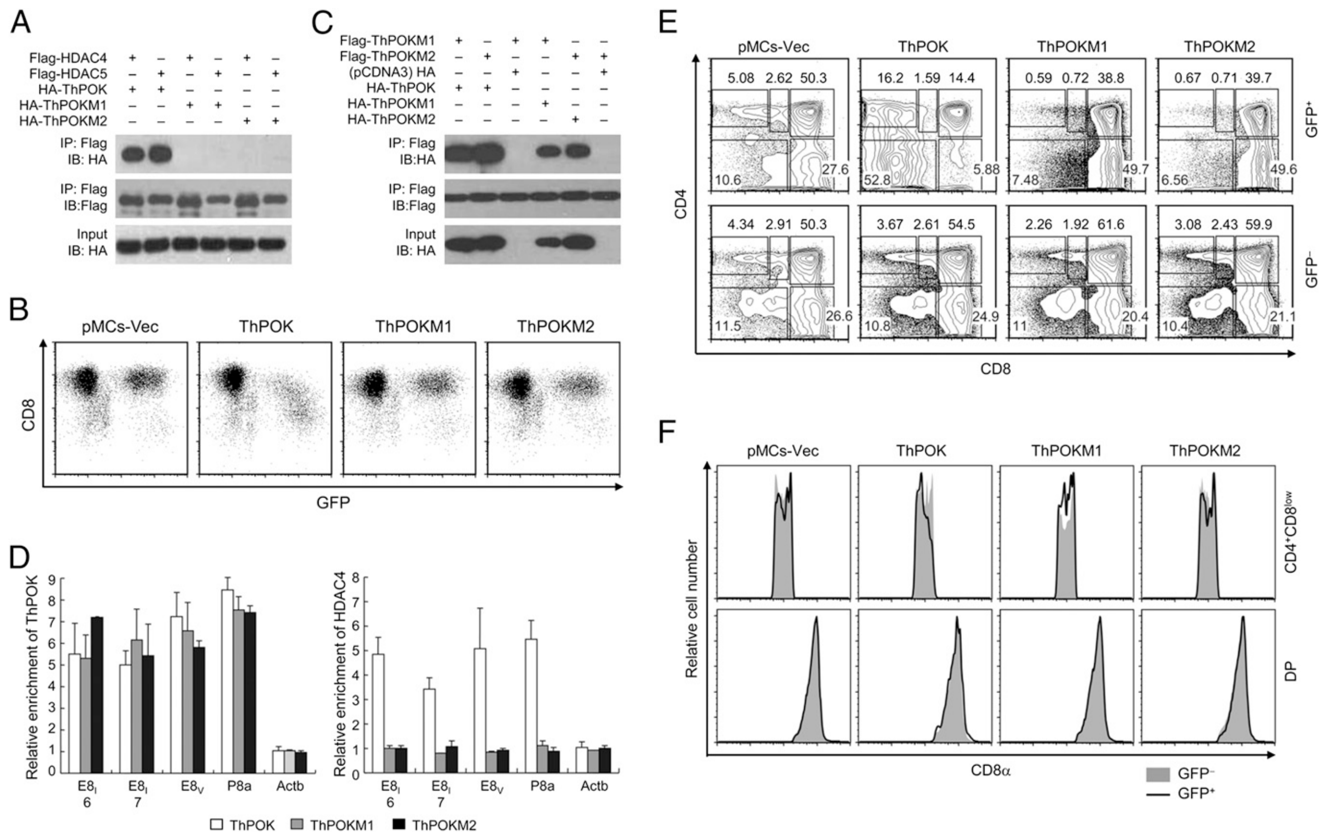

FIGURE 4. The recruitment of class II HDACs is a prerequisite of ThPOK for CD8 repression. (**A** and **C**) Coimmunoprecipitation assay was performed to examine the interaction of ThPOK mutants with class II HDACs (A) and with ThPOK (C). The results are from three independent experiments. IB, immunoblot. (**B**) ThPOKM1/M2 did not inhibit CD8 expression in transduced CD8 T cells. CD8 T cells were retrovirally transduced with the indicated constructs and subjected to FACS analysis of GFP and CD8 expression 3 d later. The data are representative of three independent experiments. (**D**) The association of ThPOK mutants and HDAC4 with *Cd8* loci from transduced CD8 T cells. Anti-ThPOK (*left*) and anti-HDAC4 (*right*) ChIP assays were performed in transduced CD8 T cells with the indicated ThPOK derivatives. Q-PCR analysis of recovered DNA from the indicated ChIP assay is shown. The data are the averages of three independent ChIP experiments, with each sample measured in duplicate (means ± SD). (**E**) Development of retroviral-transduced fetal thymocytes. Day 15.5 mouse fetal thymocytes were retrovirally transduced with various ThPOK versions and reconstituted using a deoxyguanosine-prepared fetal thymus. CD4 and CD8 surface expression was determined using FACS analysis for GFP^+ and GFP^- cells isolated from 7 to 10 d of RTOC. The numbers next to the boxes of the contour diagrams indicate the percentage of cells. The data are representative of three independent experiments. (**F**) FACS analysis of CD8 surface expression on transduced (GFP^+) or nontransduced (GFP^-) thymocytes with retroviruses containing ThPOKM1/M2 or ThPOK constructs. The data are representative of three independent experiments.

tially lower compared with their OT-1 controls (Fig. 6A, Supplemental Fig. 3A). We performed serial ChIP analysis in DP and $CD4^+CD8^{low}$ subsets from both lines. ThPOK could also be effectively recruited to *Cd8* loci in the DP cells from C8/OT-1 mice (Fig. 6B). The enrichment of HDAC4 at *Cd8* loci was unexpectedly substantial in DP cells from the same line (Fig. 6C), whereas no enrichment of ThPOK or HDAC4 could be detected in the counterparts from the OT-1 littermates (Fig. 6B, 6C). These results further suggest the ThPOK-dependent deposition of HDAC4 at *Cd8* loci. The enrichment of ThPOK and HDAC4 in the $CD4^+CD8^{low}$ subset from C8/OT-1 mice showed a similar abundance to their DP precursors and enrichment over the controls (Fig. 6D, 6E). We next analyzed the acetylation of histones H3 and H4, which were much less acetylated in the DP cells from C8/OT-1 mice than those from OT-1 littermates (Fig. 6F). Similar results were also observed in the $CD4^+CD8^{low}$ subset from C8/OT-1 mice (Fig. 6G). As a result of deacetylation, the transcription of *Cd8a* and *Cd8b* was remarkably suppressed in both subsets (especially the $CD4^+CD8^{low}$ subset) from C8/OT-1 mice (Fig. 6H, 6I).

Lineage-specific repression of *Cd4* and *Cd8* genes is associated with the repositioning of alleles close to heterochromatin (36). A three-dimensional FISH assay was performed with $CD4^+CD8^{low}$ subsets from C8/OT-1 mice and OT-1 littermates to examine the ThPOK-induced lineage-specific repression of *Cd8* genes. Consistent with changes in histone status, the association of *Cd8* loci (including one or both loci associated) with heterochromatin occurred at a much higher frequency in ThPOK-transgenic $CD4^+CD8^{low}$ cells compared with their counterparts (Fig. 6J, 6K, Supplemental Table I). These results indicate that ThPOK stably represses CD8 expression through deacetylating and repositioning *Cd8* loci near heterochromatin during the differentiation of the CD4 lineage.

Cd8 *loci are more accessible in the absence of ThPOK during CD4 T cell differentiation*

We used ThPOK-deficient mice to further define the role of ThPOK in *Cd8* silencing during the differentiation of CD4 T cells. The deposition of HDAC4 in $CD4^+CD8^{low}$ subsets was impaired in the absence of ThPOK (Fig. 7A). Similarly, the acetylation of histones H3 and H4 at the studied regions increased substantially in cells from $ThPOK^{-/-}$ mice (Fig. 7B). The *Cd8a* and *Cd8b* genes produced more abundant transcripts, which enhanced the levels of surface CD8 on $CD4^+CD8^{low}$ cells when ThPOK was ablated (Fig. 7C, 7D, Supplemental Fig. 3B). These results indicate the reversion of *Cd8* loci from a less to a more accessible status following ThPOK deficiency. Indeed, CD8 alleles associate less frequently

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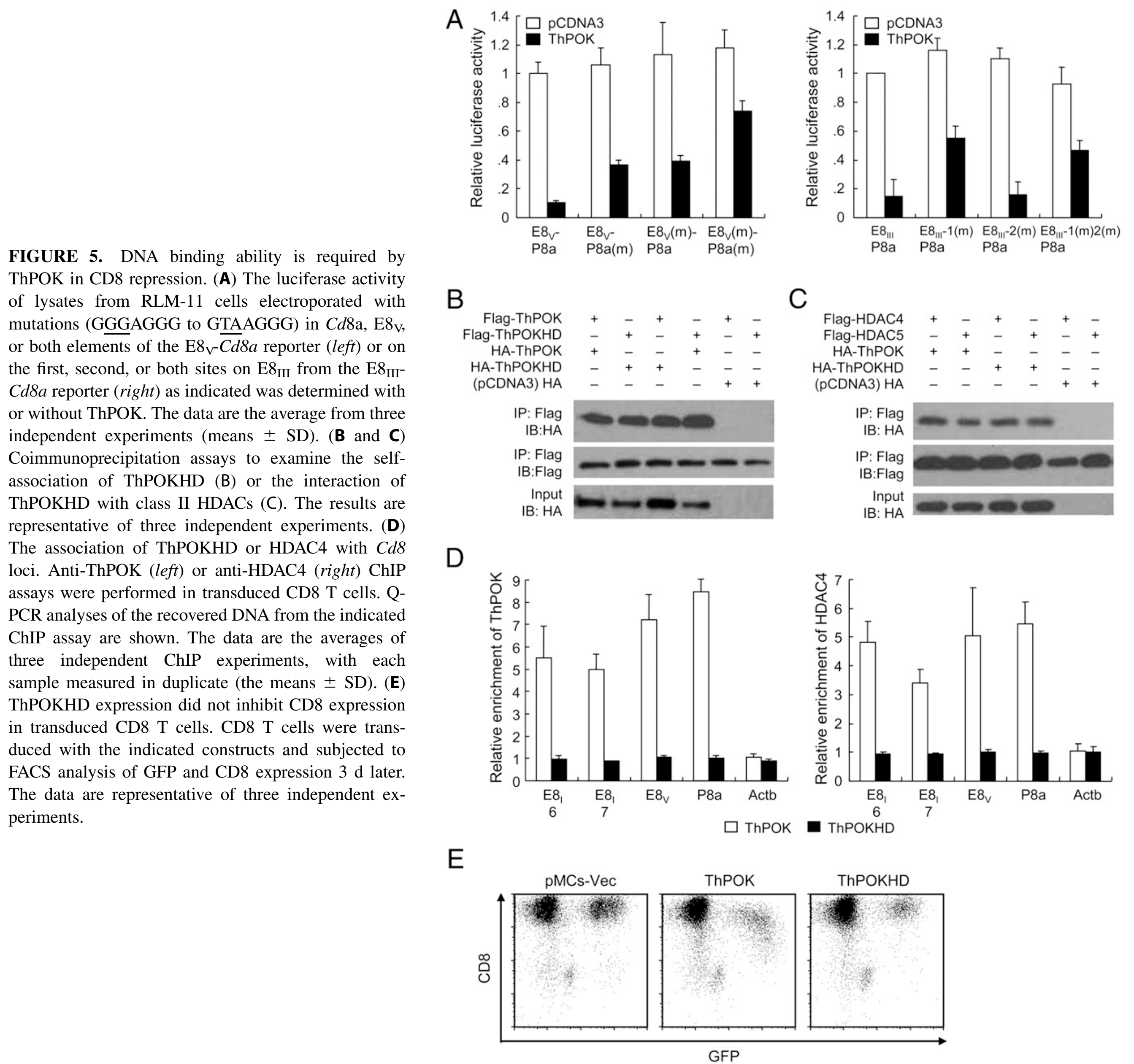

FIGURE 5. DNA binding ability is required by ThPOK in CD8 repression. (**A**) The luciferase activity of lysates from RLM-11 cells electroporated with mutations (GGGAGGG to GTAAGGG) in *Cd*8a, $E8_V$, or both elements of the $E8_V$-*Cd8a* reporter (*left*) or on the first, second, or both sites on $E8_{III}$ from the $E8_{III}$-*Cd8a* reporter (*right*) as indicated was determined with or without ThPOK. The data are the average from three independent experiments (means ± SD). (**B** and **C**) Coimmunoprecipitation assays to examine the self-association of ThPOKHD (B) or the interaction of ThPOKHD with class II HDACs (C). The results are representative of three independent experiments. (**D**) The association of ThPOKHD or HDAC4 with *Cd8* loci. Anti-ThPOK (*left*) or anti-HDAC4 (*right*) ChIP assays were performed in transduced CD8 T cells. Q-PCR analyses of the recovered DNA from the indicated ChIP assay are shown. The data are the averages of three independent ChIP experiments, with each sample measured in duplicate (the means ± SD). (**E**) ThPOKHD expression did not inhibit CD8 expression in transduced CD8 T cells. CD8 T cells were transduced with the indicated constructs and subjected to FACS analysis of GFP and CD8 expression 3 d later. The data are representative of three independent experiments.

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with heterochromatin in intermediate $CD4^+CD8^{low}$ cells from *ThPOK*$^{-/-}$ mice, as demonstrated by three-dimensional FISH (Fig. 7E), indicating that lineage-specific repression of *Cd8* genes is disturbed in the absence of ThPOK. Therefore, the ThPOK-mediated repression of *Cd8* genes was required for the stable silencing of CD8 and exclusive expression of the CD4 surface marker on the CD4 lineage cells.

Discussion

In this study, we demonstrated that ThPOK specifically associated with *Cd8* loci and functioned as a negative regulator of *Cd8* genes during differentiation of the CD4 lineage. Following ThPOK-mediated deposition of class II HDACs, CD8 alleles were deacetylated and repositioned near heterochromatin. Consequently, CD8 expression was initially repressed and eventually stably silenced, and the expression of the *Cd4* gene was exclusively maintained in the CD4 lineage.

ThPOK is acknowledged as a key regulator of the CD4/CD8 lineage choice, although it was first identified as a transactivator of type I mouse collagen. The DNA targeting sequences recognized by ThPOK are not well defined beyond a general preference for guanine-rich sequences (23). ThPOK associates with *Cd4* and *ThPOK* silencers to eliminate the negative effect of RUNX proteins through the formation of a self-regulation loop in the CD4 lineage (4). In the present study, we report that ThPOK associates with multiple sites spanning *Cd8* loci, encompassing all five *Cd8* enhancers and the *Cd8a* promoter, and functions as a negative regulator of *Cd8* genes during CD4 T cell differentiation. ThPOK associated with *Cd8* elements via the GGGAGGG consensus sites and other less conserved sequences; the DNA binding ability was indispensable for ThPOK-induced CD8 repression. Given the redundancy of *Cd8* enhancers, comprehensive binding might be important for the effective repression of CD8 during the differentiation of CD4 lineage cells.

Normally, ThPOK is expressed during the transition from DP to $CD4^+CD8^{low}$ thymocytes, and the physiological role of ThPOK could explain the relatively moderate effect of transgenic ThPOK on CD8 mRNA expression in DP cells, where the ectopically

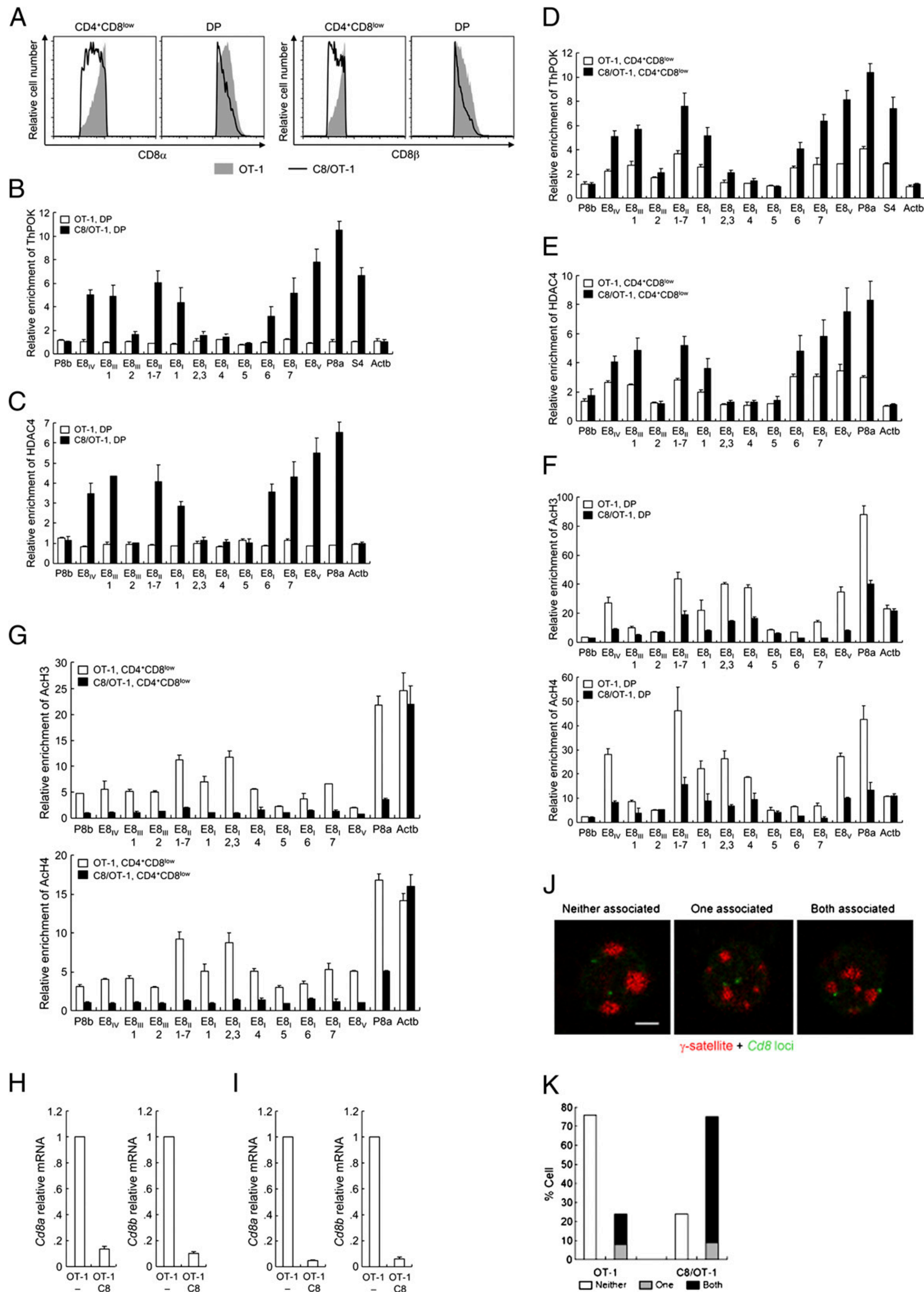

FIGURE 6. CD8 expression is attenuated in the presence of transgenic ThPOK during the differentiation of CD4 T cells. (**A**) FACS analysis of CD8α and CD8β expression in indicated C8/OT-1 and OT-1 subsets. Subsets were gated using anti-CD4 and CD8α Abs. Data are representative of three independent experiments. (**B**–**G**) Enrichment of indicated proteins at the *Cd8* loci was analyzed using a ChIP assay with anti-ThPOK (B, D), anti-HDAC4 (C, E), anti–Ace-H3 and anti–Ace-H4 (F, G) Abs or control IgG in sorted subsets from ThPOK-OT-1 double transgenic mice (C8/OT-1) and their OT-1 counterparts. The data (means ± SD) are the average of three independent ChIP experiments, with each sample measured in duplicate. (**H** and **I**) Q-PCR analysis of the *Cd8* mRNA levels (*Cd8a* and *Cd8b*) in DP (H) and $CD4^+CD8^{low}$ subsets (I) from C8/OT-1 and OT-1 mice were presented as the abundance relative to that of *Hprt*. The results (means ± SD) are from three independent experiments. (**J**) Analysis of the position of CD8 alleles relative to heterochromatin. The nuclear position of CD8 alleles (*green*) relative to γ-satellite DNA (*red*, as a marker for heterochromatin) was detected using a three-dimensional FISH assay and defined as being associated with neither, one, or both alleles. A single optical section with both alleles visible is shown in each image. Scale bar, 2 μm. (**K**) The percentage of cells with heterochromatin-associated CD8 alleles in $CD4^+CD8^{low}$ cells isolated from C8/OT-1 and OT-1 mice is shown.

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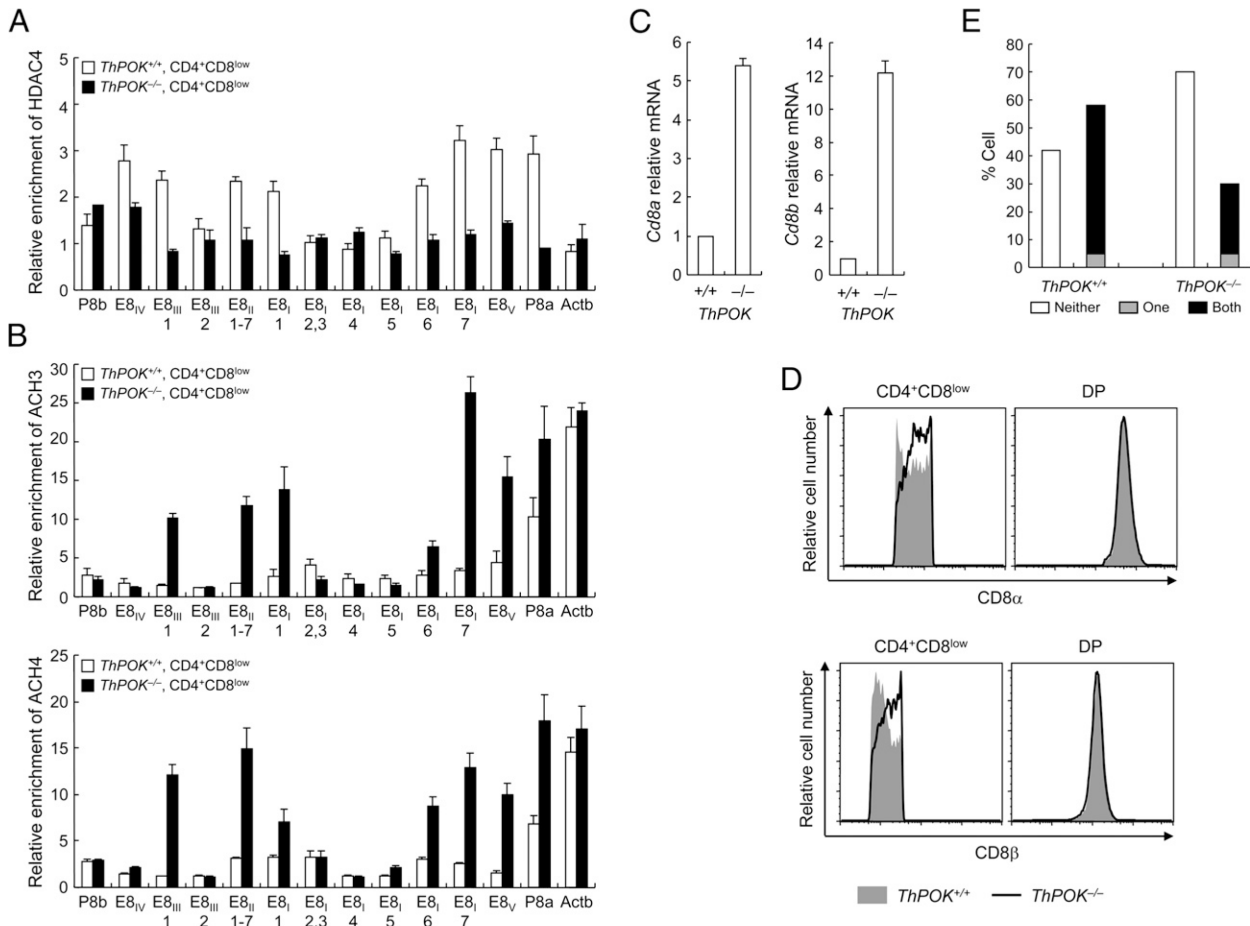

FIGURE 7. *Cd8* loci are more accessible in the absence of ThPOK during CD4 T cells development. (**A** and **B**) The relative abundance of HDAC4 (A) and Ace-H3/H4 (B) at *Cd8* loci in sorted CD4$^+$CD8^{low} thymocytes from *ThPOK*$^{+/+}$ and *ThPOK*$^{-/-}$ mice were assessed using ChIP assays with the indicated Abs. Data (means ± SD) are the average of three independent experiments with each sample measured in duplicate. (**C**) Q-PCR analysis of the *Cd8* mRNA levels in sorted CD4$^+$CD8^{low} thymocytes from *ThPOK*$^{+/+}$ and *ThPOK*$^{-/-}$ mice is presented as the abundance relative to that of *Hprt*. The results (means ± SD) are from three independent experiments. (**D**) FACS analysis of both CD8α and CD8β expression on indicated subsets from *ThPOK*$^{-/-}$ and *ThPOK*$^{+/+}$ mice. Subsets were gated using anti-CD4 and CD8α Abs. Data are representative of three independent experiments. (**E**) Analysis of the position of CD8 alleles relative to heterochromatin using a three-dimensional FISH assay in the absence of ThPOK. The percentage of cells with CD8 alleles associated with heterochromatin in CD4$^+$CD8^{low} intermediate cells isolated from *ThPOK*$^{+/+}$ and *ThPOK*$^{-/-}$ mice is shown.

expressed ThPOK competes with other factors to manipulate CD8 expression, factors including but not limited to IKAROS, RUNX1, and STAT5, which altogether are probably strong enough to activate almost all of the *Cd8* enhancers and promoters (except E8$_{I}$) in DP thymocytes. Moreover, inadequate decoration or a lack of partners could contribute to the inability or compromised function of overexpressed ThPOK in DN/DP cells. Notably, the reduced CD8 mRNA did not reflect well to surface CD8 expression in ThPOK-transgenic (C8) subsets, probably due to mRNA redundancy since the deletion of one copy of *Cd8* (or *Cd4*) alleles does not reflect well to surface CD8 (or CD4) expression (37, 38).

Posttranslational modifications, particularly acetylation, of nucleosomal histones are critical in the transcriptional regulation of genes. Global histone acetylation is regulated by the antagonistic activities of histone acetyltransferases and HDACs (39, 40). HDACs are generally recruited as part of multicomponent complexes to specific promoters via association with sequence-specific DNA-targeting factors. Accumulating evidence has shown that class II HDACs help mediate the transcriptional repression of genes that are crucial for differentiation, as documented for the serum response factor (41), MEF-2 in myogenesis (42), GATA-1 in erythroid differentiation (43), and the BTB/POK family proteins Bcl-6 and PLZF in hematopoietic lineage differentiation (26, 44). During the differentiation of CD4 T cells, as a BTB/POK family protein, ThPOK also recruit class II HDACs to negatively regulate CD8 expression. Notably, the ability to interact with ThPOK is required but not sufficient for HDACs to participate in ThPOK-mediated CD8 repression, as in the case of HDAC3. Moreover, the interaction between ThPOK and HDACs could be indirect since other BTB domain-containing factors recruit corepressors, such as N-CoR and SMRT, as part of multicomponent complexes that include HDACs (27). Because p300-mediated acetylation stabilizes ThPOK (45), it is possible that the recruitment of class II HDACs could also affect ThPOK stability and influence CD8 regulation by ThPOK.

Considering the essential roles of class II HDACs in ThPOK-mediated CD8 suppression both in vitro and in vivo, we think that deacetylation of CD8 alleles is the primary event attributed to CD8 silencing through ThPOK in the differentiation of CD4 SP cells. Still, we cannot rule out other chromatin modifications that might participate in ThPOK-mediated CD8 silencing in CD4 lineage differentiation. Unknown factors are involved in the downregulation of CD8 in the DP–CD4$^+$CD8^{low} transition and the upregulation of CD8 in the DN–DP transition, both of which are prior to lineage commitment to CD4 or CD8 and should be investigated in future studies to fully demonstrate how CD8 is si-

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lenced during the development of DP thymocytes into CD4 SP thymocytes.

The antagonistic interplay between ThPOK and RUNX3 is interesting. Both of them are lineage factors for the CD4 and CD8 lineages, respectively. To remove the regulation patterns imposed by each other, ThPOK and RUNX3 exert opposite influences on the same targets. Notably, RUNX3 binds to the *Cd4* silencer and the *ThPOK* silencer in the CD8 lineage, whereas ThPOK occupies these two silencers to protect them from RUNX3 in the CD4 lineage (4, 14). RUNX3 binding mediated the association of *Cd4* and *Cd8* loci, whereas ThPOK binding kept the loci apart (46). The regulation of CD8 through RUNX3 and ThPOK might be another demonstration of their antagonistic interplay on the same targets, given that RUNX3 associates with multiple sites spanning $E8_I$–$E8_V$ in CD8-expressing thymocytes, $E8_I$ in particular, and RUNX3 functions as a CD8 transactivator that promotes the acetylation of histones in target regions (9). In the present study, ThPOK was recruited extensively to the five *Cd8* enhancers, $E8_I$ and $E8_V$ in particular, and the *Cd8a* promoter, and it functioned as a negative *Cd8* regulator through the deacetylation of CD8 genes. Considering that ThPOK transduction into CD8 T cells inhibits their expression of other cytotoxic genes in addition to CD8 (16), ThPOK might regulate cytotoxic genes as *Runx3* using similar mechanisms.

The expression of the *Cd4* and *Cd8* genes correlates with the cell fate decisions of thymocytes; therefore, elucidating the transcriptional regulation of *Cd4* and *Cd8* genes might help reveal the molecular basis of lineage commitment (5). Stable repression of *Cd4* and *Cd8* genes, which is associated with the repositioning of alleles close to heterochromatin, is a central feature of T cell lineage commitment (36, 47). During the differentiation of CD4 T cells, CD8 goes through a two-step downregulation, first from TCR-signaled DP thymocytes to $CD4^+CD8^{low}$ intermediates (a common step for both CD4 and CD8 lineages), followed by long-term CD8 silencing from CD4-committed intermediates in CD4 SP cells, which is potentially fulfilled through ThPOK, as we showed that ThPOK mediated the reposition of *Cd8* genes near the heterochromatin and stably repressed *Cd8* genes during this process. As to the initial CD8 downregulation during the DP–$CD4^+$ $CD8^{low}$ transition, which can be only marginally attributed to ThPOK, other factors of greater importance, such as GATA-3 and c-Myb, might also be involved (11, 48).

In conclusion, our data highlight the role of ThPOK as a negative regulator of *Cd8* genes during the differentiation of CD4 SP thymocytes. ThPOK mediates the deacetylation and reposition of *Cd8* genes near heterochromatin through specific association with *Cd8 cis* elements and deposition of class II HDACs to *Cd8* loci. Therefore, *Cd8* genes were eventually silenced in the CD4 lineage. Our results reveal the mechanism involved in *Cd8* silencing during the commitment of CD4 SP cells and the essential roles of ThPOK and class II HDAC-induced deacetylation in this process.

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Disclosures

The authors have no financial conflicts of interest.

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