

Epigenetic *Thpok* silencing limits the time window to choose CD4⁺ helper-lineage fate in the thymus

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CD4⁺ helper and CD8⁺ cytotoxic T cells differentiate from common precursors in the thymus after T-cell receptor (TCR)-mediated selection. Commitment to the helper lineage depends on persistent TCR signals and expression of the ThPOK transcription factor, whereas a *ThPOK* cis-regulatory element, *ThPOK* silencer, represses *Thpok* gene expression during commitment to the cytotoxic lineage. Here, we show that silencer-mediated alterations of chromatin structures in cytotoxic-lineage thymocytes establish a repressive state that is epigenetically inherited in peripheral CD8⁺ T cells even after removal of the silencer. When silencer activity is enhanced in helper-lineage cells, by increasing its copy number, a similar heritable *Thpok* silencing occurs. Epigenetic locking of the *Thpok* locus may therefore be an independent event from commitment to the cytotoxic lineage. These findings imply that long-lasting TCR signals are needed to establish stable *Thpok* expression activity to commit to helper T-cell fate and that full commitment to the helper lineage requires persistent reversal of silencer activity during a particular time window.

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Introduction

Differentiation of multi-potent precursors into a particular lineage upon exposure to stimuli from external environmental cues is accompanied by the expression of lineage-specific genes together with repression of alternative lineages, a process termed as lineage specification. As a consequence of sequential exposures to differentiation signals, specific gene expression signatures that confer unique cellular functions to

differentiated cells are established (lineage commitment) (Cantor and Orkin, 2001; Murphy and Reiner, 2002; Rothenberg and Dionne, 2002). Mechanisms that guarantee the stable inheritance of established gene signatures even after multiple cell divisions enable differentiated cells to preserve their cell identity. Studies in the last decade have highlighted the crucial contributions of epigenetics in maintenance of gene expression status (Jenuwein and Allis, 2001; Probst *et al.*, 2009; Bonasio *et al.*, 2010). For instance, heterochromatin has been shown to be involved in heritable and stable gene repression (Henikoff, 2000), referred to as gene silencing, in part via relocating gene loci into specialized nuclear compartments such as the peri-nuclear lamina (Schneider and Grosschedl, 2007; Reddy *et al.*, 2008). It is thus conceivable that lineage commitment would involve epigenetic changes towards heterochromatin-like structures at many developmentally regulated genes to assure their subsequent silent state. However, how the epigenetic machinery that delivers lineage-specific epigenetic marks is linked with lineage commitment remains uncharacterized. Specifically, it is not clear whether such epigenetic machinery becomes functional upon lineage commitment or acts in parallel with lineage specification. In order to better understand an epigenetic link between environmental cues and establishment of cell identity, it is important to unravel how specific epigenetic modifications are accumulated on the relevant genes.

There are two major lineages of T lymphocytes, the CD4⁺ helper and the CD8⁺ cytotoxic cells, and they differentiate from a common precursor, the CD4⁺CD8⁺ double-positive (DP) thymocyte, after positive selection. During this selection process, only DP thymocytes that receive optimal TCR-signal strength as a result of TCR engagement with peptide-MHC complexes are allowed to further differentiate (Germain, 2002; Starr *et al.*, 2003; Singer *et al.*, 2008). When CD4⁺CD8⁺ DP thymocytes are positively selected through MHC class II molecules (MHC class II-selected thymocytes), they develop into CD4⁺CD8⁻ single-positive (SP) thymocytes that are committed to the helper lineage. On the contrary, DP thymocytes that undergo selection via MHC class I molecules with a help of CD8 co-receptors terminate CD4 expression and differentiate into CD4⁻CD8⁺ SP thymocytes committed to the cytotoxic lineage (Ellmeier *et al.*, 1999). It has been proposed that differences in TCR-signal length during positive selection instruct distinct fates in post-selection thymocytes. Several lines of evidence have shown that persistent TCR signalling is essential for development of CD4⁺ helper T cells (Sarafova *et al.*, 2005; Singer *et al.*, 2008; Adoro *et al.*, 2012). On the other hand, temporary termination of *Cd8* gene expression in post-selection thymocytes (Brugnera *et al.*, 2000) results in a disruption of TCR signals specifically in MHC class I-selected thymocytes, instructing them to become cytotoxic-lineage cells (Singer *et al.*, 2008). However, it remains obscure why long-lasting TCR signals are necessary for commitment to the helper lineage.

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Recently, it has been recognized that appropriate linkage of TCR specificity to MHC class during helper/cytotoxic lineage choice requires input from the zing finger transcription factor ThPOK (also known as cKrox), which is encoded by the *Zbtb7b* gene (hereafter referred to as the *Thpok* gene in this manuscript) (He *et al*, 2005, 2010; Sun *et al*, 2005). Gain and loss of function studies of ThPOK demonstrated a dominant role of ThPOK in acquiring a CD4⁺CD8⁻ phenotype that is independent of TCR specificity to MHC class. Thus, both MHC class I-selected and class II-selected thymocytes are redirected to alternative CD4⁺CD8⁻ helper and CD4⁻CD8⁺ cytotoxic lineages by ectopic induction or loss of ThPOK function, respectively (He *et al*, 2005; Sun *et al*, 2005). Because of its potent activity, expression of the *Thpok* gene from its two promoters, distal P1 and proximal P2, must be strictly regulated during thymocyte differentiation. During thymocyte maturation, ThPOK first appears after positive selection, increases in MHC class II-selected thymocytes, but then disappears in MHC class I-selected cells (He *et al*, 2005; Muroi *et al*, 2008). In sorting out mechanisms that control lineage- and stage-specific *Thpok* expression, previous studies have identified two essential *cis*-regulatory elements. A transcriptional silencer (referred to as the *Thpok* silencer in this manuscript) is essential to control helper lineage-specific expression of *Thpok* (He *et al*, 2008; Setoguchi *et al*, 2008). On the other hand, a transcriptional enhancer in the proximal region of the gene plays an essential role in increasing *Thpok* expression at later maturation stages in MHC class II-selected thymocytes (Muroi *et al*, 2008). Interestingly, conditional ablation of ThPOK function from peripheral CD4⁺ T cells indicated that extra-thymic expression of ThPOK is still necessary to maintain CD4⁺ helper T-cell identity (Wang *et al*, 2008). Furthermore, retroviral transduction of ThPOK into fully differentiated peripheral CD8⁺ T cells can activate helper lineage-related genes as well as repress cytotoxic-related genes, albeit only to limited extent (Jenkinson *et al*, 2007). These findings indicate that the helper lineage-specific *Thpok* expression established in the thymus must be sustained in the peripheral T cells to maintain their lineage identity. However, although roles of *cis*-regulatory elements in regulating *Thpok* expression during lineage commitment in thymus have begun to be characterized (Taniuchi, 2009), it remains unknown how the established state of the *Thpok* gene, either active or repressive, is stably maintained in differentiated T cells.

To address this point, we used genetic approaches that enabled us to modify *Thpok* silencer activity. We show that a silencer-dependent deposition of repressive epigenetic marks establishes a repressive state that is inherited independently of the silencer in peripheral CD8⁺ T cells via inhibiting the two promoters by different mechanisms. Furthermore, we provide evidence showing that such epigenetic *Thpok* silencing can also occur in CD4⁺ helper-lineage cells when silencer activity persists after positive selection. These findings suggest that epigenetic mechanisms that lock the *Thpok* locus can take place even in MHC class II signalled cells if the silencer activity is not properly terminated. Thus, our findings strongly suggest that, for appropriate helper-lineage choice by MHC class II-selected cells, reversal of the *Thpok* silencer upon receipt of TCR signals must persist for a certain time interval to avoid

epigenetic sealing of the *Thpok* gene. We thus propose that such continuous counter-silencing by long-lasting TCR signals is necessary to establish stable *Thpok* expression, hence to fully commit to helper T-cell fate.

Results

Epigenetic modifications in the *Thpok* gene

There is now compelling evidence that developmentally regulated genes receive dynamic modifications of local chromatin structure (Bonasio *et al*, 2010), and in particular, heterochromatin-like structures are known to be involved in stable gene repression (Henikoff, 2000). We therefore examined how the chromatin structure at the *Thpok* locus is altered during T-cell development. Since tri-methylation at two distinct histone H3 lysine residues, lysine 4 and lysine 27 (H3K4me3 and H3K27me3), has been shown to mark active and repressive states, respectively (Bernstein *et al*, 2005), of the target genes, we performed chromatin immunoprecipitation (ChIP)-on-chip analysis of distinct T-cell subsets. Accumulation of the repressive H3K27me3 mark at the distal P1 promoter and its upstream region was detected from immature CD4⁻CD8⁻ DN thymocytes onward in cell subsets that do not express *Thpok*, while it was not present at a significant level at the proximal P2 promoter (Figure 1A and C). In contrast, H3K27me3 deposition at the distal P1 promoter was replaced by active H3K4me3 marks in cells expressing *Thpok*, such as CD4⁺CD8⁻ SP thymocytes, along with accumulation of H3K4me3 at the proximal P2 promoter (Figure 1A). Interestingly, a comparison of the *Thpok* silencer-deficient and wild-type *Thpok* loci by analytical ChIP assays revealed that the loss of the *Thpok* silencer resulted in not only a decrease in H3K27me3 marks but also an increase in H3K4me3 marks around the P1 promoter in DP thymocytes (Figure 1B and C). Similar changes resulting from loss of the silencer were also observed at the P2 promoter, albeit to a lesser extent (Figure 1B and C). These results indicate that the silencer is involved in the initial deposition of H3K27me3 marks as well as in preventing premature H3K4me3 loading prior to positive selection.

Another known epigenetic modification involved in gene silencing is DNA methylation (Jones, 2012). We therefore next compared the DNA methylation status of the *Thpok* gene in CD4⁺ and CD8⁺ peripheral T cells. We tested 21 CpG islands derived from the *Thpok* silencer, distal P1 promoter, proximal P2 promoter and proximal enhancer (PE) and only three regions (S-4, 1-4 and 1-5) exhibited slightly increased DNA methylation in CD8⁺ T cells compared to CD4⁺ T cells (Figure 2A). Thus, our analyses did not detect any clear differences in the DNA methylation status of active versus repressed *Thpok* genes, although they could not formally exclude involvement of DNA methylation at other sites in repression of the *Thpok* gene.

To further examine roles of epigenetic modifications in *Thpok* repression, we treated CD8⁺ T cells with Trichostatin A (TSA) or 5-aza-2'-deoxycytidine (5-AzaC), inhibitors of histone deacetylases (HDAC) and DNA methyltransferase, respectively. Three days after TSA treatment, *Thpok* expression in activated CD8⁺ T cells was significantly increased specifically from the P1 promoter, albeit at a level still well below that in CD4⁺ T cells. On the other hand, 5-AzaC induced a slight derepression of the *Thpok* gene mainly

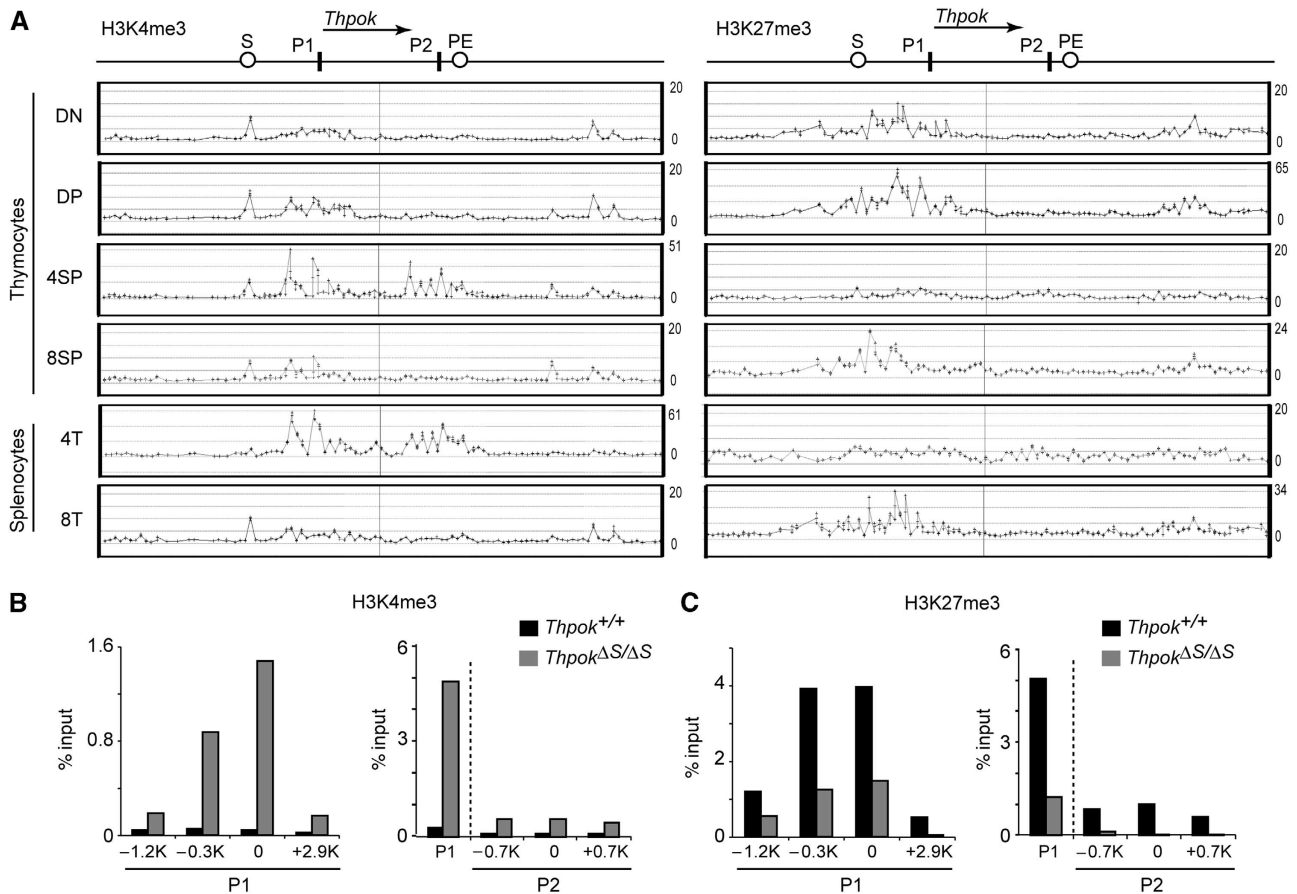


Figure 1 Changes of histone H3 modifications pattern in the *Thpok* gene during T-cell development. (A) Association of histone H3 K4 tri-methylation (H3K4me3) and histone H3 K27 tri-methylation (H3K27me3) in the *Thpok* locus. The signal intensities of individual oligonucleotide probe as revealed in a ChIP-on-chip assay in the indicated T-cell subsets are shown along with a schematic structure of the *Thpok* gene (top). Positions of the silencer (S), proximal enhancer (PE), distal P1 promoter (P1) and proximal P2 promoter (P2) are indicated. One representative result of two experiments is shown. (B, C) Comparison of H3K4me3 (B) and H3K27me3 (C) patterns at the region around the distal P1 and proximal P2 promoters between silencer-sufficient (*Thpok*^{+/+}) and -deficient (*Thpok*^{ΔS/ΔS}) DP thymocytes determined by analytical ChIP assays. Positions of each region analysed are shown as distance from the transcriptional start site in the P1 or P2 promoter-derived *Thpok* mRNA. In the panel shown at the right, the P1 promoter is also included as a reference. Data are from one of two independent experiments.

from the P2 promoter in a variegated manner (Figure 2B and C). Although it was not clear whether these inhibitors induced *Thpok* de-repression directly or indirectly, these results, along with the distinct H3K27me3 deposition pattern, suggested that different mechanisms are likely to be involved in preventing the activity of the two promoters for stable repression of the *Thpok* gene in CD8⁺ T cells.

Conditional deletion of the *Thpok* silencer from CD8-lineage cells

Our result showed that lineage-specific chromatin structures are established at the *Thpok* locus and that this correlates with the presence of the *Thpok* silencer. To further investigate the relevance of such epigenetic modifications for the regulation of *Thpok* expression, we wished to test whether the repressive state is maintained independently of the silencer in differentiated CD8⁺ T cells, as was observed at the *Cd4* locus (Zou *et al*, 2001). To this end, we have generated a *Thpok*^{Sfl} allele in which an 800-bp region including the entire *Thpok* silencer sequence was flanked by two loxP sites (Supplementary Figure 1). The excision of the silencer in precursor DP thymocytes by *Cd4-Cre* transgene resulted in the

loss of peripheral CD8⁺ T cells, as was previously observed in the case of germline deletion of the silencer (Figure 3A; Setoguchi *et al*, 2008). On the contrary, deletion of the *Thpok* silencer in developing CD4⁻CD8⁺ SP thymocytes by *E81-Cre* transgene (Maekawa *et al*, 2008), which is expressed after the cells have downregulated the CD24 surface marker, did not impair development of CD8⁺ T cells (Figure 3A). Genotype analyses by Southern blot confirmed that the excision of the silencer by *E81-Cre* was not at a detectable level in total thymocytes, but was almost complete in and restricted to CD8⁺ cells among splenic T cells (Figure 3B). The level of *Thpok* mRNA in these silencer-deficient CD8⁺ T cells was slightly elevated, but still <20% of that in CD4⁺ T cells (Figure 3C). Thus, the conditional removal of the *Thpok* silencer from post-selection thymocytes differentiating into the cytotoxic lineage did not cause a massive de-repression of the *Thpok* gene. Interestingly, only P1-derived *Thpok* transcripts were detected in the CD8⁺ T cells from *Thpok*^{Sfl/Sfl};*E81* mice (Figure 3D), whereas germline deletion of the silencer from a *Thpok*^{Sflp} reporter allele led to de-repression of the *gfp* gene from both the P1 and P2 promoters in CD8⁺ T cells (Figure 3E).

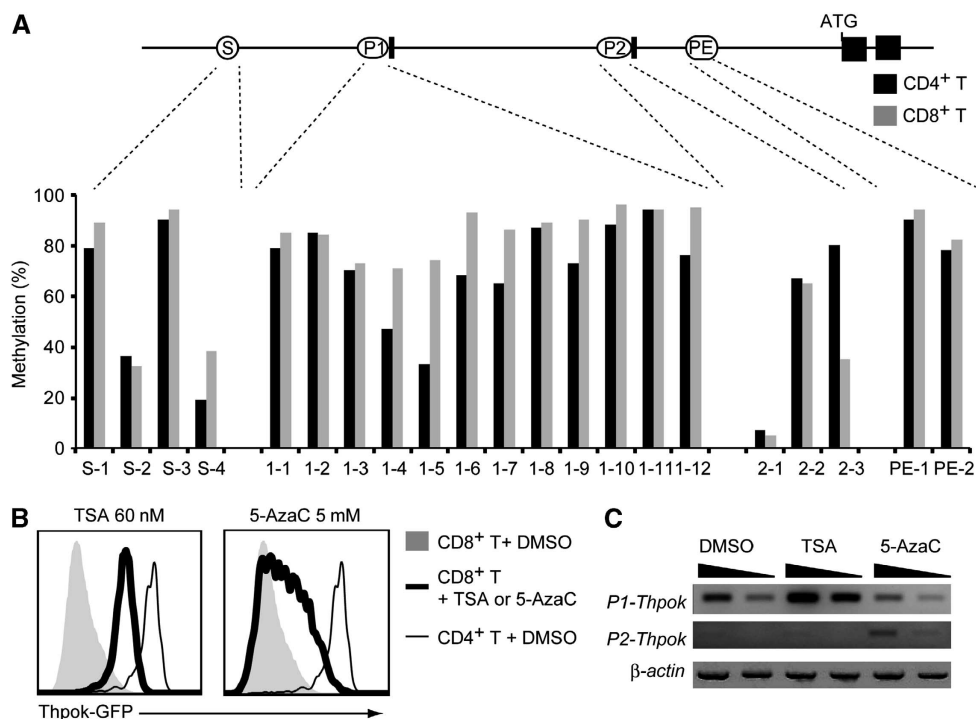


Figure 2 Comparison of DNA methylation status of the *Thpok* locus in CD4⁺ and CD8⁺ T cells. (A) DNA methylation status in CD4⁺ and CD8⁺ T cells analysed by methylation-specific PCR at selected CpG islands located within the silencer (S), distal P1 promoter (P1), proximal P2 promoter (P2) and proximal enhancer (PE) are shown as percentages. (B) Effects of chemical inhibitors of histone deacetylases, Trichostatin A (TSA), and DNA methyltransferase, 5-aza-2'-cytidine (5-AzaC), on the *Thpok* repression. Histograms showing *Thpok*-GFP expression from the *Thpok*^{gfp} allele in activated CD8⁺ T cells 3 days after treatment with either inhibitor in culture. Expression of GFP in control CD4⁺ T cells is shown as a thin line for reference. (C) Semi-quantitative RT-PCR for the P1 promoter- and the P2 promoter-derived *Thpok* mRNA after inhibitor treatment is shown with the β-actin mRNA as control. Wedges indicate 1:3 dilution of template. Data are from one of two independent experiments. Source data for this figure is available on the online supplementary information page.

We next examined the effect of deleting the *Thpok* silencer from fully differentiated CD8⁺ T cells prepared from spleen. As previously reported (Setoguchi *et al*, 2009), *Thpok*-GFP expression was detected in activated CD8⁺ T cells from the *Thpok*^{gfp} allele, but not the *Thpok*^{gfp:ΔPE} allele lacking the PE, after *in vitro* TCR stimulation (Figure 3F), indicating that the PE is necessary for *Thpok* expression in activated CD8⁺ T cells. Both P1 and P2 promoter-derived *Thpok* mRNA was detected in activated CD4⁺ T cells, however, activated CD8⁺ T cells contained only the P1-derived *Thpok* mRNA (Figure 3G). When these CD4⁺ and CD8⁺ T cells from *Thpok*^{Sfl/Sfl} mice were transduced with retroviral vectors encoding Cre recombinase, the silencer region was efficiently excised within 48 h (Figure 3H). However, the amount of *Thpok* mRNA in activated CD8⁺ T cells was not elevated after removal of the silencer (Figure 3I) and the P2 promoter-derived *Thpok* mRNA remained undetectable (Figure 3J). These results demonstrate that sequential epigenetic modifications at the *Thpok* locus during commitment to the CD8⁺ cytotoxic lineage in the thymus established an epigenetically repressed state that is maintained in peripheral CD8⁺ T cells independently of the silencer.

Effect of enhanced *Thpok* silencer activity by increasing its copy number

It is known that increasing the copy number of a *cis*-regulatory region can enhance its function (Herr and Gluzman, 1985). Indeed, three copies of the *Thpok* silencer in a reporter plasmid repressed *luciferase* reporter expression

more efficiently than one copy in a transfection assay (Supplementary Figure 2). We then wished to examine whether and how an increase in silencer copy number at the endogenous *Thpok* gene would affect its expression. To this end, we have generated a *Thpok*^{gfp:3S} allele, in which three tandem copies of 202–401 core silencer sequences were inserted into the *Thpok*^{gfp} reporter allele by sequential gene targeting into ES cells of the *Thpok*^{+/sfl} genotype (Supplementary Figure 3). Expression of GFP from the *Thpok*^{gfp:3S} allele was analysed in *Thpok*^{+/gfp:3S} mice, in which normal CD4⁺ T-cell development is supported by the half dosage of ThPOK produced from the wild-type allele. The induction of GFP in the CD69⁺ TCRβ^{hi} thymocyte subset from the *Thpok*^{gfp:3S} allele was lower than from the control *Thpok*^{gfp} allele with respect to both expression levels and the percentage of GFP⁺ cells (Figure 4A). Interestingly, GFP expression was lost in some CD4⁺ T cells but was only reduced in other CD4⁺ cells, thus exhibiting variegated repression rather than the uniform repression that was observed with the *Thpok*^{gfp:ΔPE} allele (Figure 4A). Since reduced GFP expression was not observed in non-T cells (Figure 4A), the effect of increasing silencer copy number was likely to be CD4-lineage specific rather than a general phenomenon in any types of cells.

To examine how impaired ThPOK expression due to the insertion of three copies of the *Thpok* silencer affects T-cell development, we have generated a *Thpok*^{3S} allele by targeting the same 3S mutation into the *Thpok* allele (Supplementary Figure 3). In *Thpok*^{3S/3S} mice, the percentages of CD4⁺ CD8⁻

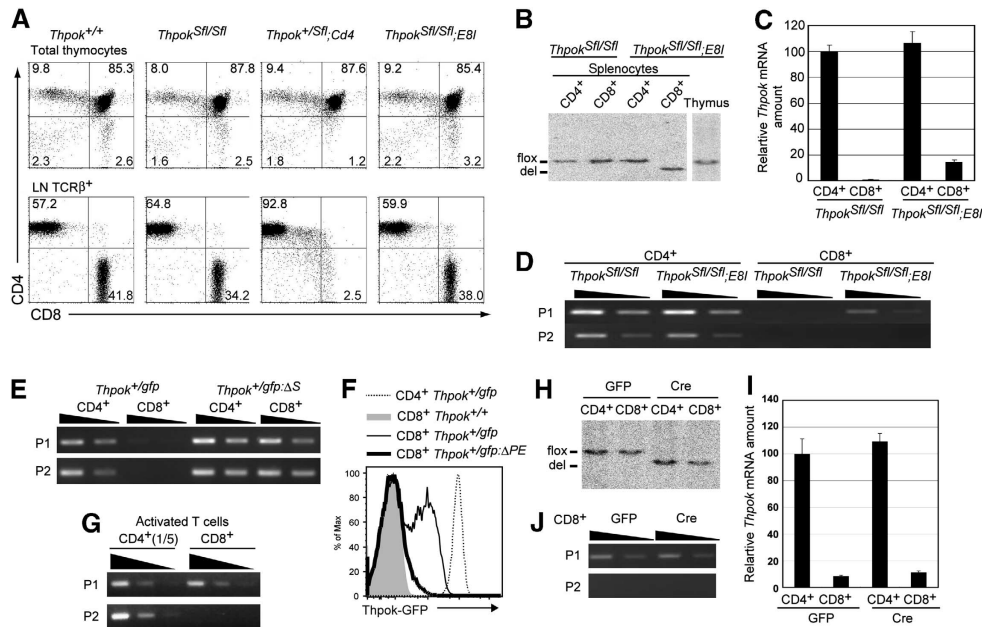


Figure 3 Effects of conditional removal of the *Thpok* silencer in CD8-lineage cells on *Thpok* expression. (A) Dot plots showing CD4 and CD8 expression in total thymocytes and lymph node TCRβ⁺ T cells from mice of the indicated genotypes. Numbers in dot plots indicate the percentage of cells in each quadrant. (B–D) Genotype analyses by Southern blot (B), relative *Thpok* mRNA amounts (C) and semi-quantitative RT-PCR for the P1 promoter- and the P2 promoter-derived *Thpok* mRNA (D) in CD4⁺ and CD8⁺ T cells from the indicated mice. (E, G) Semi-quantitative RT-PCR for the P1 promoter- and the P2 promoter-derived *gfp* mRNA from the *Thpok*^{gfp} and the silencer-deficient *Thpok*^{gfp:ΔS} allele in CD4⁺ and CD8⁺ T cells (E), and for the P1- and P2-derived *Thpok* mRNA in activated CD4⁺ and CD8⁺ T cells (G). (F) Histogram showing Thpok-GFP expression in the indicated activated T cells of indicated genotypes. (H–J) Genotype analyses by Southern blot (H), relative *Thpok* mRNA amount (I) and semi-quantitative RT-PCR for the P1 promoter- and the P2 promoter-derived *Thpok* mRNA (J) in activated CD4⁺ and CD8⁺ T cells from *Thpok*^{Sfl/Sfl} mice transduced with control retroviral vector (GFP) or vector encoding Cre recombinase (Cre). Wedges in semi-quantitative RT-PCR assay indicate 1:3 dilution of template, except in (G) (1:5 dilutions). Data are from one of at least two independent experiments. Source data for this figure is available on the online supplementary information page.

cells were reduced both in the thymus and in peripheral T-cell pools (Figure 4B). In *Thpok*^{3S/gfp} mice in which ThPOK protein was produced only from the *ThPOK*^{3S} allele, the decrease in the CD4⁺ T-cell subset became more evident with emergence of CD8⁺ T cells expressing GFP. Given that GFP expression from the *Thpok*^{gfp} allele is specific to MHC class II-selected cells (Muroi *et al*, 2008), these CD8⁺ GFP⁺ cells are likely to be re-directed MHC class II-selected cells. Indeed, when we examined the differentiation of MHC class II-selected cells in mice with no surface expression of MHC class I molecules, cells with CD4[−]CD8[−], CD4⁺CD8⁺ and CD4[−]CD8⁺ phenotype emerged in the peripheral lymphoid organs of *Thpok*^{3S/3S} and *Thpok*^{3S/gfp} mice whereas those cells were absent in control mice (Figure 4C). These observations indicated that enhanced *Thpok* silencer activity resulting from an increase in its copy number in the *Thpok* gene perturbed development of CD4⁺ T cells via inhibiting *Thpok* expression in a variegated manner.

Possible epigenetic *Thpok* silencing in CD4-lineage cells

Variegated gene expression has been shown to be a characteristic of gene silencing mediated by heterochromatin-like structures (Weiler and Wakimoto, 1995). Given the variegated GFP expression from the *Thpok*^{gfp:3S} allele in CD4⁺ T cells and the epigenetic control of the *Thpok* locus that results in a silencer-independent maintenance of *Thpok* silencing in CD8-lineage cells, we next addressed whether an epigenetic repressive state, which is maintained independently of the silencers, is similarly established at

the *Thpok*^{gfp:3S} allele in CD4⁺ helper-lineage cells. To this end, we have generated a *Thpok*^{gfp:3S/FRT} allele in which the three copies of the silencer can be excised upon expression of Flp recombinase (Figure 5A; Supplementary Figure 3). GFP expression from the *Thpok*^{+/gfp:3S/FRT} allele after *in vitro* TCR stimulation was still low in CD4⁺ T cells and was not detected in activated CD8⁺ T cells (Figure 5B). In addition, both P1 and P2 promoter-derived *gfp* mRNA was reduced in resting CD4⁺ T cells from *Thpok*^{+/gfp:3S/FRT} mice (Figure 5C). Consistent with these observations, ChIP assays demonstrated an increase and decrease in H3K27me3 and H3K4me3 marks, respectively, at the region nearby the silencer on the *Thpok*^{gfp:3S/FRT} allele, compared to those on the wild-type *Thpok* allele in the same CD4⁺ T cells of *Thpok*^{+/gfp:3S/FRT} mice (Figure 5D). Thus, chromatin structures at the *Thpok*^{gfp:3S/FRT} allele in CD4⁺ T cells were similar to those at the wild-type *Thpok* allele in CD8⁺ T cells. To examine whether GFP expression is restored if the three copies of the silencer are removed, we transduced CD4⁺ T cells with a retroviral vector encoding Flp recombinase to obtain a population of cells in which the FRT-flanked silencers were efficiently excised (Figure 5E). Interestingly, the mean fluorescent intensity of GFP and *gfp* mRNA levels were not elevated after excision of the 3 × silencer (Figure 5F and G), confirming that the silent state at the *Thpok*^{gfp:3S/FRT} allele in CD4⁺ helper-lineage cells could be maintained in the absence of the three *Thpok* silencer copies. These genetic results clearly demonstrate that epigenetic *Thpok* silencing could be established not only in cytotoxic-lineage cells but

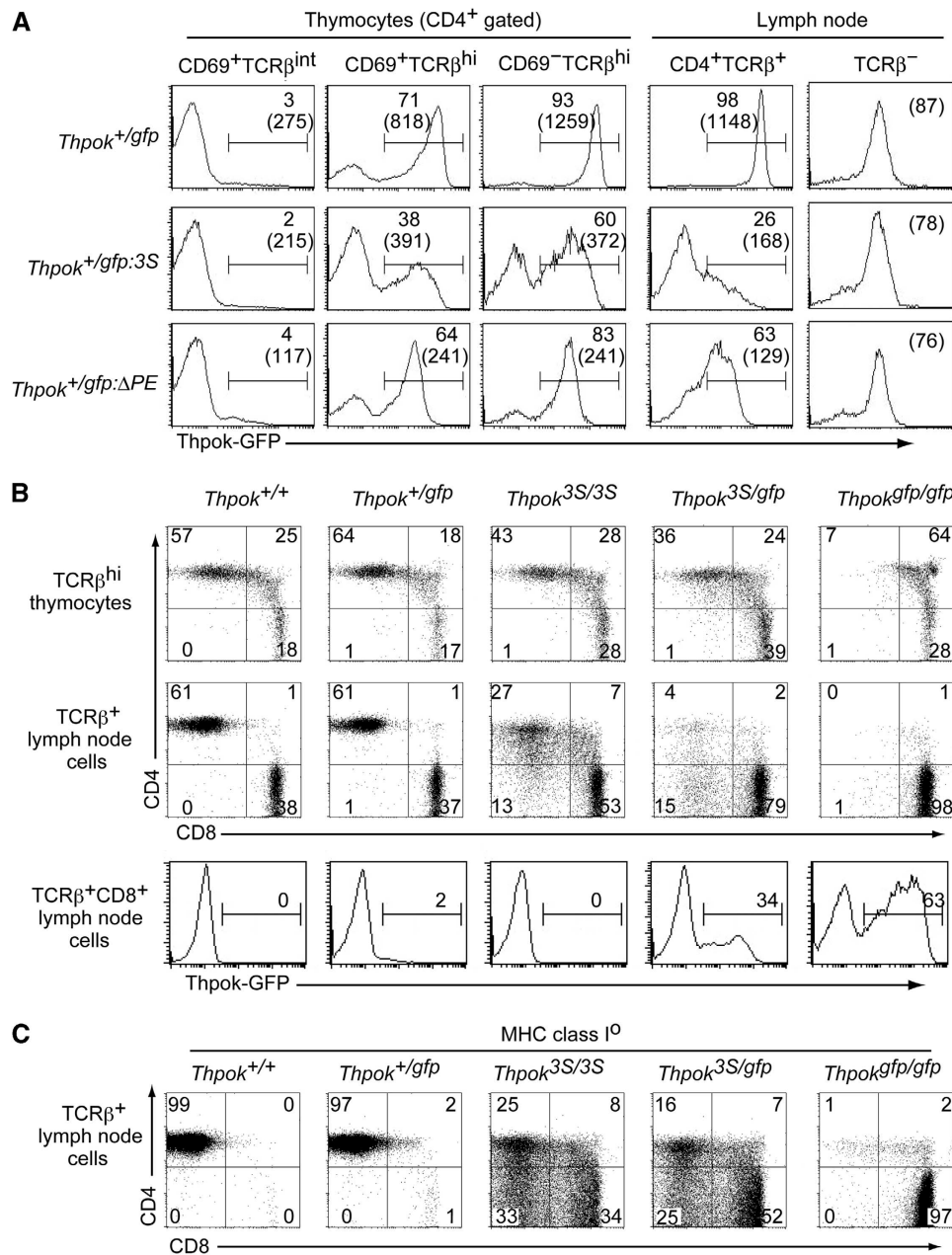


Figure 4 Redirection of MHC class II-selected cells due to impaired *Thpok* expression by increase in the silencer copy number from one to three in the *Thpok* locus. (A) Histograms showing *Thpok*-GFP expression in the indicated T-cell subsets of *Thpok*^{+/gfp}, *Thpok*^{+/gfp:3S} and *Thpok*^{+/gfp:ΔPE} mice. Numbers in the histogram indicate the percentage of GFP-positive cells, and numbers in parenthesis indicate the mean fluorescence intensity of GFP in GFP-positive cells. Notably, GFP expression in non-T cells of all three genotypes is same. (B) Dot plots showing CD4 and CD8 expression in mature (TCRβ^{hi}) thymocytes and lymph node (LN) TCRβ⁺ cells from mice of the indicated genotypes. Histograms show *Thpok*-GFP expression from the *Thpok*^{gfp} allele in TCRβ⁺ CD8⁺ LN cells. Numbers in dot plots and histograms indicate the percentage of cells in each quadrant and the percentage of GFP-positive cells. (C) Dot plots showing CD4 and CD8 expression in lymph node TCRβ⁺ cells from mice of the indicated genotypes in the absence of cell surface expression of MHC class I molecules due to a genetic ablation of β2-microglobulin. Data are one representative of at least three independent experiments.

also in helper-lineage cells when the silencer activity is strengthened by increasing its copy number.

Distinct requirement for the Runx complex function in reversible *Thpok* repression versus establishment of irreversible *Thpok* silencing

We next addressed which functional sites in the *Thpok* silencer are important to establish epigenetic *Thpok* silencing by a 'knock-in' mutagenesis assay (Figure 6A; Supplementary

Figure 3). Whereas removal of the 242–401 core silencer sequences from the *Thpok*^{gfp} allele led to full GFP depression in CD8⁺ T cells, repression was restored by reinsertion of wild-type 242–401 sequences (Figure 6B), confirming that junctional sequences created by ligation of the tested fragment did not affect silencer activity. Given the controversy about whether or not Runx sites are essential for *Thpok* silencer activity (He *et al*, 2008; Hedrick, 2008; Setoguchi *et al*, 2008), we introduced targeted mutations

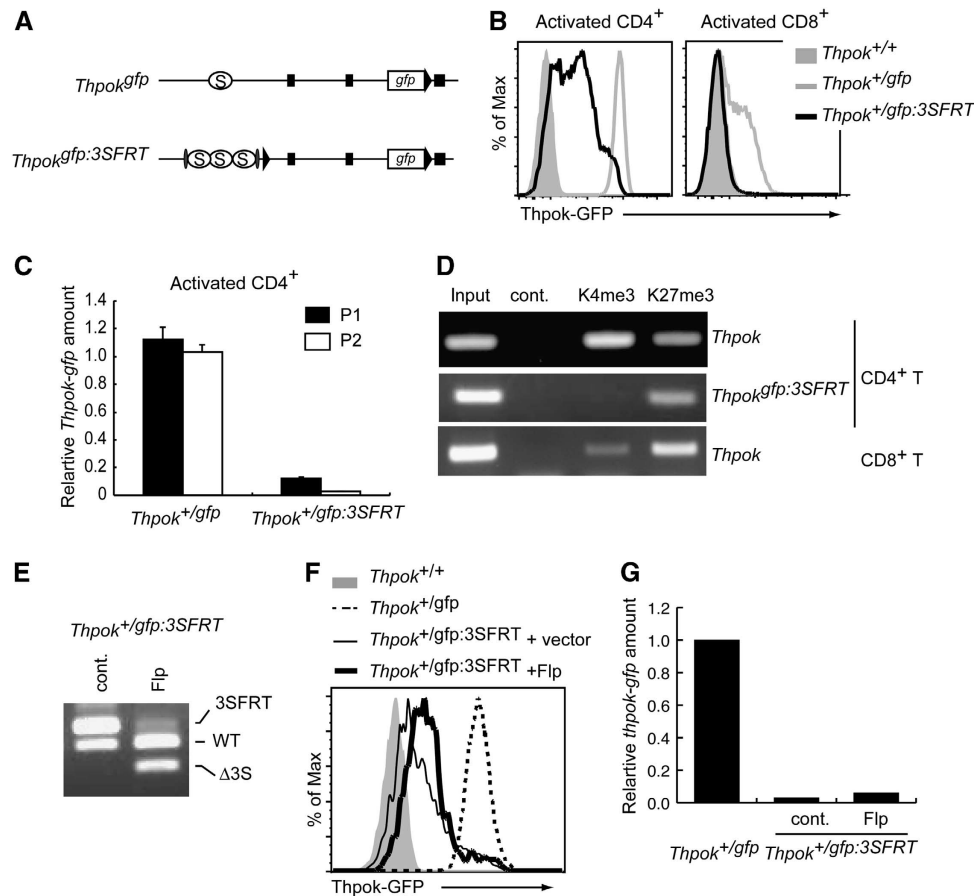


Figure 5 Epigenetic *Thpok* silencing of the *Thpok^{gfp:3SFRT}* allele in CD4⁺ helper T cells. (A) Schematic structure of *Thpok^{gfp}* and *Thpok^{gfp:3SFRT}* alleles. Black triangles, grey ovals, black boxes and open circles marked with S represent loxP sites, FRT sites, exons and the silencer region, respectively. (B) Histograms showing Thpok-GFP expression in the indicated activated T cells with indicated genotypes. (C) Relative amounts of the P1 promoter- and the P2 promoter-derived *gfp* mRNA from the *Thpok^{gfp}* and *Thpok^{gfp:3SFRT}* alleles in activated CD4⁺ T cells. (D) ChIP assay showing associations of histone H3K4 tri-methylation (K4me3) and histone H3K27 tri-methylation (K27me3) with the silencer region of the wild-type *Thpok* or *Thpok^{gfp:3SFRT}* allele in CD4⁺ and CD8⁺ T cells from *Thpok^{+/gfp:3SFRT}* mice. (E–G) Effect of removal of three copies of the silencers from the *Thpok^{gfp:3SFRT}* allele in activated CD4⁺ T cells transduced with control vector (cont.) or vector encoding Flp recombinase (Flp). Genotyping analyses by DNA-PCR (E), histogram showing Thpok-GFP expression (F) and relative *Thpok* mRNA amounts (G) from *Thpok^{gfp:3SFRT}* in the presence or absence of silencers and those from *Thpok^{gfp}* or *Thpok* as controls (F). Data shown are one representative of at least two independent experiments. Source data for this figure is available on the online supplementary information page.

into the two Runx sites and generated a *Thpok^{gfp:242–401RM}* reporter allele harbouring 242–401RM mutant sequences. Strikingly, upon loss of the two Runx sites, high GFP expression was induced in most of CD8⁺ T cells, although a small proportion of the cells expressed GFP at a low level (Figure 6B). ChIP assays with DP thymocytes from *Thpok^{+/gfp:242–401RM}* mice showed that binding of Runx complexes to the *Thpok^{gfp:242–401RM}* allele was severely reduced compared to the wild-type *Thpok* allele (Figure 6C). Furthermore, the ratio of H3K4me3 to H3K27me3 depositions at the *Thpok^{gfp:242–401RM}* allele in CD8⁺ T cells of *Thpok^{+/gfp:242–401RM}* mice was reversed compared to wild type (Figure 6D). This finding is inconsistent with previous results detecting substantial silencer activity in a mutant silencer fragment lacking Runx sites in a transgenic reporter expression assay (He *et al*, 2008). Given that silencer activity was enhanced by increasing its copy number, we next tested whether insertion of three copies of the 242–401RM mutant silencer restores *Thpok* silencing by generating a *Thpok^{gfp:3xRM}* allele (Figure 6A). In the *Thpok^{+/gfp:3xRM}* mice, GFP expression was lost in about three-quarter of

CD8⁺ T cells (Figure 6B), along with restoration of Runx binding to the silencer to some extent (Figure 6C), although a low amount of GFP expression was still detected in about 25% of those cells. This result suggests that Runx complexes might be recruited to the *Thpok^{gfp:3xRM}* allele through interaction with other silencer binding proteins. Indeed, the percentage of CD8⁺ T cells de-repressing GFP increased when the dosage of the *Bcl11b* gene, whose product, Bcl11b, binds to the *Thpok* silencer independently of Runx sites (Tanaka H, unpublished observation), was halved (Figure 6E). Taking into account the observed restoration of silencer activity by increasing copies of the mutant silencer lacking Runx sites from one to three, it is possible that integration of the transgene in tandem as multiple copies would influence the expression pattern of the reporter transgene and mask any effect of mutation in the *cis*-regulatory regions tested. In any cases, these genetic results clearly indicate that binding of Runx complexes to the *Thpok* silencer is essential for recruitment of nuclear protein complexes that catalyse epigenetic modifications to establish epigenetic *Thpok* silencing.

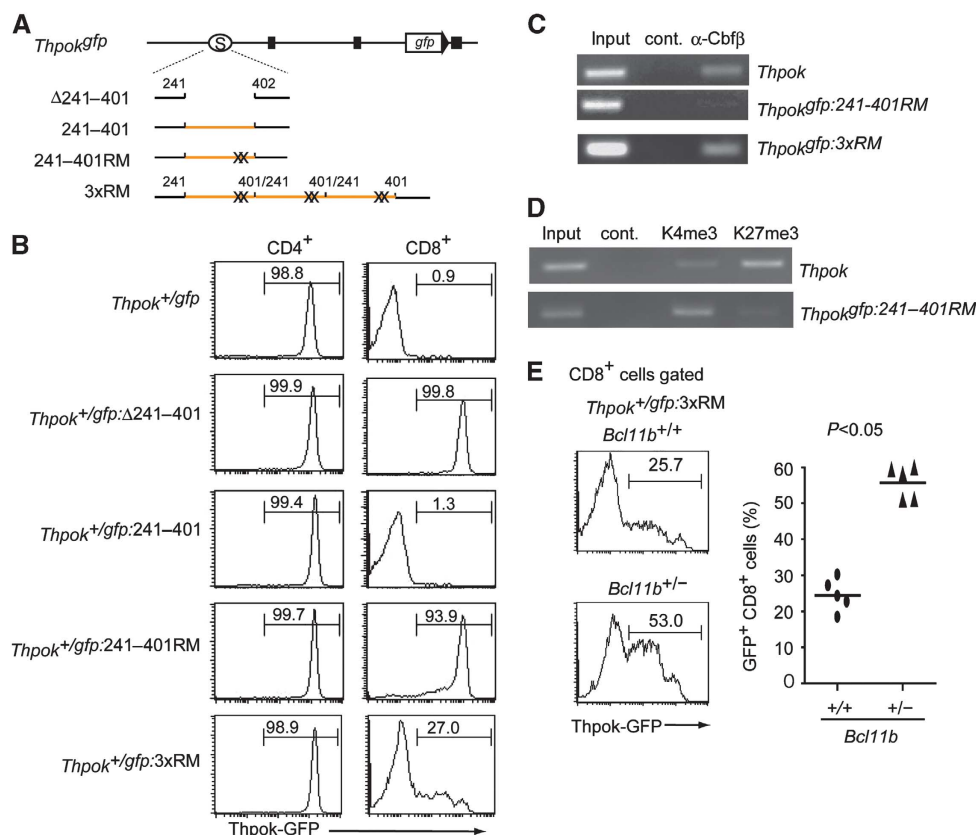


Figure 6 Requirement for Runx complex binding to the silencer in establishment of epigenetic *Thpok* silencing. (A) Schematic structures of mutant *Thpok^{gfp}* reporter alleles. Black boxes, open circles marked with S, orange lines and X represent exons, the silencer region, the 242–401 core silencer sequences and mutations at Runx sites, respectively. (B) Histograms showing Thpok-GFP expression in the indicated T-cell population from mice of the indicated genotypes. Numbers in the histogram indicate the percentage of GFP-positive cells. (C, D) ChIP assays showing binding of Runx/Cbfb complexes to the silencer region on the *Thpok*, the *Thpok^{gfp}:242-401RM* or the *Thpok^{gfp}:3xRM* allele in DP thymocytes (C), histone H3K4 tri-methylation (K4me3) and histone H3K27 tri-methylation (K27me3) to the silencer region on the *Thpok* or *Thpok^{gfp}:242-401RM* allele in CD8⁺ T cells (D). Data are one representative of at least three individual experiments. (E) Histograms showing Thpok-GFP expression in CD8⁺ T cells of *Thpok^{+/gfp}:3xRM* mice with full (*Bcl11b^{+/+}*) or half dosage (*Bcl11b^{+/-}*) of the *Bcl11b* gene. Numbers in the histogram indicate the percentage of GFP-positive cells. Graph at the right shows results with individual mice. Source data for this figure is available on the online supplementary information page.

However, although *Thpok* repression in precursor DP thymocytes can be reversible, our analyses detected Runx complex binding to the silencer already in those cells (Figure 6C), raising the possibility of a distinct mode of action by Runx in repressing *Thpok* at two developmental stages. We therefore examined whether Runx complexes are functionally involved in *Thpok* repression by analysing *Thpok-gfp* reporter expression in precursor thymocytes. While low, but significant, GFP expression was induced in about 14% of CD24^{hi} TCR^{int} pre-selection thymocytes of *Thpok^{+/gfp}: ΔS* mice, the percentage of GFP⁺ precursors was only ~4% in *Thpok^{+/gfp}:242-401RM* mice (Figure 7A). In contrast, in the cytotoxic-lineage committed CD8⁺CD24^{low}TCR^{hi} mature thymocytes, GFP expression was quite similar between *Thpok^{+/gfp}: ΔS* and *Thpok^{+/gfp}:242-401RM* mice (Figure 7A), although lower GFP expression was observed in a small proportion of *Thpok^{+/gfp}:242-401RM* cells, as was also observed in peripheral mature CD8⁺ T cells (Figure 6B). Thus, the requirement for Runx recognition sites for silencer activity could differ before and after positive selection.

The VWRPY penta-peptide sequence is present in Runx proteins of all species examined and serves as a platform to recruit TLE/Groucho co-repressors to Runx proteins. Indeed, we recently reported that *Thpok* silencing in cytotoxic-lineage

T cells was impaired in *Runx1 ^{ΔV / ΔV} :Runx3 ^{ΔV / ΔV}* mice in which the VWRPY motif was removed from both Runx1 and Runx3 proteins (Seo *et al*, 2012). We therefore next examined *Thpok* expression during differentiation of *Runx1 ^{ΔV / ΔV} :Runx3 ^{ΔV / ΔV}* thymocytes, and found that it remained repressed in precursor DP thymocytes whereas it was de-repressed in mature CD8-lineage thymocytes in a variegated manner (Figure 7B). These results suggest that regulation of the *Thpok* silencer function by Runx complexes may differ depending on developmental stage, with reversible repression in pre-selection precursors versus establishment of epigenetic silencing in post-selection thymocytes developing into the CD8 lineage.

Discussion

In this study, we examined how chromatin structure at the *Thpok* locus is modified during T-cell development. We observed that patterns of representative active and repressive histone modifications, H3K4me3 and H3K27me3, at the distal P1 promoter are dynamically altered and this correlated with promoter activity. Our genetic approach also uncovered a strong correlation between epigenetic modifications established during lineage commitment in the thymus

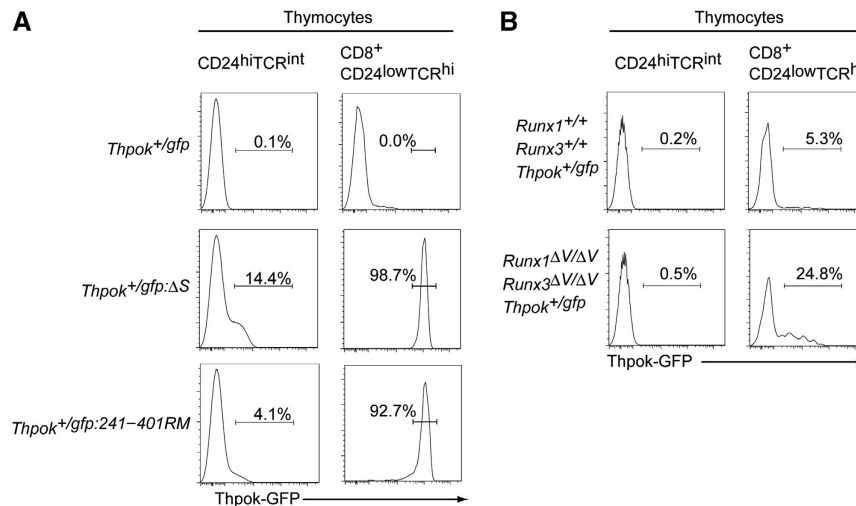


Figure 7 Runx complexes are nearly dispensable for *Thpok* repression in pre-selection thymocytes. (A) Histograms showing *Thpok*-GFP expression in pre-selection (CD24^{high}TCR^{int}) thymocytes and cytotoxic-lineage committed (CD8⁺CD24^{low}TCR^{hi}) mature thymocytes from mice of the indicated genotypes. Numbers in the histogram indicate the percentage of GFP-positive cells. (B) Histograms showing *Thpok*-GFP expression in pre-selection (CD24^{high}TCR^{int}) thymocytes and mature CD8-lineage (CD8⁺CD24^{low}TCR^{hi}) mature thymocytes from control mice (*Runx1*^{+/+};*Runx3*^{+/+};*Thpok*^{+/gfp}) and mutant mice (*Runx1*^{ΔV/ΔV};*Runx3*^{ΔV/ΔV};*Thpok*^{+/gfp}) that lack the VWRPY motif, a platform for recruiting TLE/Groucho co-repressors, from both Runx1 and Runx3 proteins. Numbers in the histogram indicate the percentage of GFP-positive cells. These results highlight a requirement for Runx proteins in establishment of epigenetic *Thpok* silencing that occurs specifically in post-selection thymocytes.

and the maintenance of lineage-specific *Thpok* expression in the periphery. In particular, once silencer-dependent chromatin modifications were completed, the *Thpok* gene remained silenced independently of the *Thpok* silencer in CD8⁺ T cells, similar to the situation reported for the *Cd4* locus (Zou *et al*, 2001). Furthermore, by generating mutant *Thpok* alleles harbouring three copies of the *Thpok* silencer, we showed that similar epigenetic *Thpok* silencing could be established in CD4⁺ T cells if the silencer activity is strengthened. Although the precise mechanism by which silencer activity is modified by increasing its copy number remains unclear, three silencers might potentially increase the rate of epigenetic modifications during a certain time period or extend the duration of a developmental window during which the silencer functions. Whatever the mechanisms, silencing by the three-copy silencers was not observed in B cells, strongly suggesting that this is not an artefact, and supporting its physiological relevance in the potency of epigenetic *Thpok* silencing in helper-lineage cells. Thus, even though this observation originated from an artificially modified *Thpok* locus, the potential for epigenetic *Thpok* silencing even in CD4⁺ T cells indicates that the nuclear machinery that establishes chromatin structures adequate for the silencer-independent maintenance of *Thpok* repression is not only present but also potentially functional even in cells differentiating towards the helper lineage. Thus, our findings indicate that epigenetic processes that stably silence the *Thpok* gene can occur independently of commitment to the cytotoxic lineage.

It has been proposed that proper development of MHC class II-selected thymocytes into the helper lineage requires persistence of TCR signals (Sarafova *et al*, 2005; Singer *et al*, 2008). However, it has been unclear how the length of TCR signalling can be linked with nuclear events controlling CD4/CD8 lineage choice, such as of *Thpok* expression, which clearly depends on TCR signals (Kappes, 2010).

Our findings suggest that silencer-dependent chromatin modifications could potentially occur in all post-selection thymocytes unless the *Thpok* silencer is functionally inactivated. This silencer-mediated mode of regulation is likely to sequentially and gradually modify chromatin towards heterochromatin-like structures on the *Thpok*^{3S} allele, even in cells developing into the helper lineage. The gradual nature of the epigenetic modification process presumably allows the *Thpok* locus to retain plasticity during a certain time window after the *Thpok* silencer is initially inactivated under TCR signals. It is therefore an intriguing possibility that the *Thpok* locus can undergo reverse chromatin modifications, leading towards the silenced state upon reactivation of the silencer. Physiologically, this could be induced by disruption of TCR signals, leading to a failure to produce enough ThPOK to support differentiation into CD4⁺ T cells. Along with previous finding that appropriate development of CD4⁺ helper T cells depends upon production of the appropriate amount of ThPOK protein (Muroi *et al*, 2008), our results suggest that reversal of the *Thpok* silencer activity must persist for a certain length of time to prevent accumulation of repressive epigenetic marks that convert the *Thpok* locus into a firmly repressed state. Thus, it is possible that chromatin alterations leading to stable silencing of the *Thpok* gene could serve as a mechanism that restricts a developmental time window to select the helper lineage by MHC class II-selected thymocytes. Interestingly, epigenetic regulatory mechanisms have been shown to be involved in determining stage-specific developmental potency in neural precursor cells (Hirabayashi and Gotoh, 2010), suggesting conserved features of epigenetic regulation in limiting the time available to multi-potent precursors for selecting a specific fate. In this case, sequential and temporally reversible epigenetic modifications at the *Thpok* locus would also serve as the mechanism underlying quantitative

conversion of TCR-signal length into *Thpok* expression level. Precedent for such a mechanism exists in the *Flowering Locus C* gene in *Arabidopsis*, where the amount of H3K27me3 mirrors the duration of exposure to cold temperature (Huff and Zilberman, 2012). It is therefore important not only to further characterize the epigenetic machinery responsible for stable inheritance of *Thpok* silencing, but also to address its quantitative correlation with TCR signals.

Our results also indicated that the *Thpok* silencer-mediated regulation generates distinct repressive states at distinct developmental stages in terms of reversibility to an active state. In reporter transgene expression assays, *Thpok* silencer activity is detected from immature DN thymocyte to DP thymocyte stages (Muroi *et al*, 2013), consistent with the accumulation of H3K27me3 marks at the *Thpok* locus in those cells. Thus, the *Thpok* silencer already exerts its functions at the *Thpok* locus in uncommitted immature thymocytes to prevent its premature expression. However, the activation of the *Thpok* gene upon exposure to developmental cues that direct the cell towards the CD4 lineage indicates that the repressive state of the *Thpok* gene in such precursors retains the potential to readily revert into an active state. This concept is further supported with *Thpok* de-repression to the level sufficient to re-direct MHC class I-selected cells to CD4⁺ T cells by deletion of the silencer in precursors by *Cd4-Cre*. On the contrary, the conditional removal of the silencer in developing CD8 SP thymocytes by *E81-Cre* transgene failed to induce high levels of *Thpok* expression. These findings suggest that alternation of chromatin structures towards stable and heritable epigenetic *Thpok* silencing might begin only after positive selection. Of note, the transcriptional silencer in the *Cd4* locus can establish heritable epigenetic *Cd4* silencing only in post-selection thymocytes developing into the cytotoxic lineage, even though repression of the *Cd4* gene in immature DN thymocytes by the same silencer is reversible (Zou *et al*, 2001; Taniuchi *et al*, 2002b). Thus, epigenetic silencing at both *Thpok* and *Cd4* loci commonly occurs only after positive selection. Given that the activity of both *Thpok* and *Cd4* silencers requires Runx complex binding (Taniuchi *et al*, 2002a), loading of proteins specifically responsible for modifying chromatin leading to a heritable silent state might depend on both positive selection and Runx complexes. However, our results showed a distinct requirement for Runx complexes in reversible *Thpok* repression versus stable *Thpok* silencing. In contrast, Runx complexes are likely to be commonly essential for *Cd4* repression in DN thymocytes and epigenetic *Cd4* silencing in CD8-lineage cells (Taniuchi *et al*, 2002a). In addition, the VWRPY motif in Runx proteins is less necessary for epigenetic silencing at the *Thpok* than the *Cd4* loci (Seo *et al*, 2012). Thus, there are both common and unique features of *Thpok* and *Cd4* silencer-mediated gene repression. It will be important to understand the molecular basis that conveys commonality as well as uniqueness to these two silencers.

Another interesting finding was that the *Thpok* silencer regulates two promoters in the *Thpok* gene through different mechanisms. The presence of H3K27me3 repressive marks and a higher DNA methylation frequency in *Thpok* non-expressing cells were obvious only at the distal P1 promoter. However, despite the presence of such well-established repressive epigenetic marks, the P1 promoter retains the

potential for reactivation in CD8⁺ T cells upon TCR stimulation, a process enhanced by HDAC inhibitor treatment. On the contrary, the proximal P2 promoter is stably silenced after activation of CD8⁺ T cells despite limited deposition of such repressive epigenetic marks. Given a slight increase in P2-derived *Thpok* mRNA upon treatment with 5-AzaC, the regulation of the P2 promoter is likely to involve uncharacterized epigenetic mechanisms that might cooperate with DNA methylation. Furthermore, leaky expression of P1 promoter-, but not the P2 promoter-, derived *Thpok* mRNA in resting CD8⁺ T cells of *Thpok^{Sfl/Sfl};E81* mice indicates that the processes that inactivate promoter function might be completed earlier at the P2 than at the P1 promoter. It will be important to address how a single silencer can inactivate two promoters located within an ~6 kb interval by distinct manners.

Collectively, our results shed light on the nuclear regulatory event that would link TCR signals with the epigenetic regulation of *Thpok* expression. Nevertheless, uncovering properties of the molecular switch for *Thpok* silencer activity under TCR signals is extremely crucial to understand the underlying mechanisms of the link between signal length and lineage choice.

Materials and methods

Mice

β 2-microglobulin-deficient mice (Koller *et al*, 1990), *Thpok^{Sfl}* mice (Muroi *et al*, 2008), *E81-Cre* transgenic mice (Maekawa *et al*, 2008), *Bcl11b*-deficient mice (Wakabayashi *et al*, 2003) and *Runx1^{ΔV/ΔV};Runx3^{ΔV/ΔV}* mice (Seo *et al*, 2012) have been described. All mice were bred and housed in the animal facility at RIKEN RCAI, and all experiments were performed in accordance with institutional guidelines for animal care.

Generation of mice harbouring mutations in the *Thpok* locus

Construction of each targeting vector for generating mutant *Thpok* alleles is described in Supplementary Methods. The targeting vectors that were targeted to the *Thpok^{Sfl}* allele were transfected into an ES cell clone harbouring the *Thpok^{+/Sfl}* genotype (Muroi *et al*, 2008), otherwise a *wild-type* ES cell line was used. Culture of ES cells and removal of the *neo^r* gene in ES cells by transient transfection of a Cre recombinase expression vector were performed as previously described (Muroi *et al*, 2008). Homologous recombination in ES cells was first screened by PCR or Southern blot. In order to determine whether the *wild-type Thpok* or *Thpok^{Sfl}* allele underwent homologous recombination with the targeting vector, ES clones were transduced with the retroviral vector encoding Cre recombinase and then screened by PCR that detected recombination between the loxP site downstream of the *gfp* gene and the loxP sites upstream of the *neo^r* gene (Supplementary Figure 3). Detection of the above recombination event was interpreted as confirmation of homologous recombination on the *Thpok^{Sfl}* allele. To establish a mouse strain containing the *Thpok^{PEfl}* mutant allele, we used ES clone harbouring *Thpok^{PEfl/Sfl}* genotype that was obtained during generation of the *Thpok^{ΔPE}* mouse strain (Muroi *et al*, 2008).

Flow cytometry and cell sorting

Thymus, spleen and lymph nodes were removed from mice at 4–8 weeks of age and were used to make single-cell suspensions. Cells were stained with the following FITC, PE, PerCP or APC-conjugated antibodies purchased from BD Biosciences: CD4 (RM4-5), CD8 (53-6.7), CD69 (H1.2F3) and TCR β (H57-597). For staining of intracellular Gata3, cells were fixed and permeabilized with Cytotfix/perm solution (BD Biosciences) and stained with AlexaFluor 647-conjugated anti-Gata3 antibody (BD Biosciences). Multi-colour flow cytometry data were collected with a FACSCalibur or FACSCanto II (BD-Biosciences) and were analysed with BD CellQuest (BD Biosciences) or Flowjo (Tree Star) software. T-cell subsets were sorted using a FACSAria (BD Biosciences).

Cell culture with TSA and 5-AzaC

Sorted CD8⁺ T cells were stimulated with 2 µg/ml of plate-bound CD3ε antibody (BD Biosciences) and 2 µg/ml of soluble anti-CD28 antibody (BD Biosciences) in optimized DMEM (KOHJIN BIO) supplemented with 10% FBS for 48 h and were then maintained in medium supplemented with rIL-2 (20 U/ml). Twenty-four hours after initial stimulation, cells were treated with 60 nM of TSA (T8552, Sigma), or 5 mM 5-AzaC (014-20943, Wako Chemical) for 3 days and then analysed.

RNA isolation and RT-PCR

Total cellular RNA was extracted using Trizol Reagent (Invitrogen), and treated with RNase-free DNase I (Invitrogen) prior to reverse transcription in order to eliminate contaminating genomic DNA. cDNA was synthesized from total RNA using the SuperScript^{III} First Strand Synthesis System (Invitrogen). Quantitative RT-PCR was performed using the ABI/PRISM 7000 sequence detection system (Applied Biosystems) with an internal fluorescent TaqMan probe for the *Thpok* transcript and SYBR Green (Takara) for the *gfp* transcript. The amounts of *Thpok* and *gfp* mRNA were normalized against *Hprt1* mRNA and β -*actin* mRNA expression, respectively. Primers and a probe for quantitative RT-PCR for *Thpok* were previously described (He *et al*, 2005). Sequences of all other primers are shown in Supplementary Table.

ChIP and tiling array

For a ChIP-on-chip experiment, chromatin DNA from 10 million cells was immune-precipitated with anti-H3K4me3 (ab1012, ABCOM) or anti-H3K27me3 (07-449, Millipore) antibody, and then amplified twice by LM-PCR according to manufacturer's protocol (Agilent). We used custom microarrays generated by Agilent that tiled through the *Thpok* locus as previously described (Muroi *et al*, 2008). Probe hybridization and scanning of oligonucleotide array data were performed according to manufacturers' protocol (Agilent). Feature Extraction software and Genomic Workbench (Agilent) were used for data analysis. For analytical ChIP assay, 1–10 million cells were immune-precipitated with anti-Cbfb (Naoe *et al*, 2007) antibody.

DNA methylation assay

Methylation-specific PCR (Herman *et al*, 1996) was performed to analyse DNA methylation status by outsourcing (Towa Environment Science Co. Ltd). In brief, the MethylSEQR Bisulfite Conversion

Kit (Applied Biosystems) was used for bisulfite conversion assay. Prediction of DNA methylation sites such as CpG islands within target regions and design of methylated primer sets and unmethylated primer sets were performed using MethylPrimer Express (Applied Biosystems). DNA methylation status was quantitatively analysed by real-time PCR, and was calculated as percentage of methylated Cytidine in methylated Cytidine + unmethylated Cytidine.

Retroviral transduction of T cells

A retroviral vector encoding Flp recombinase was constructed by insertion of a Flp encoding cDNA, which was PCR amplified from the pCAGGS-FLPe vector (GENE BRIDGES), into pMXs-IRES-hNGFR vector (a gift from Dr Yamashita at Chiba University). Transduction of retroviral products into T cells was performed as previously described (Naoe *et al*, 2007). Due to low recombination efficiency of the vector encoded Flp recombinase, we repeated Flp transduction three times over 2 days, sorted hNGFR-positive cells, then reactivated the cells, repeated the transductions, and sorted hNGFR-expressing cells 2 days after the last transduction.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: HT and TN performed phenotypic analyses of mice and molecular biology experiments with the help of RC and CM. SM generated all mutant mouse strains. WS analysed *Runx1*^{ΔV/ΔV};*Runx3*^{ΔV/ΔV} mice. KR provided important experimental material. IT designed the study and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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