STAT6-mediated displacement of polycomb by trithorax complex establishes long-term maintenance of *GATA3* expression in T helper type 2 cells

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Polycomb group (PcG) and trithorax group (TrxG) complexes exert opposing effects on the maintenance of the transcriptional status of the developmentally regulated Hox genes. In this study, we show that activation of STAT6 induces displacement of the PcG complex by the TrxG complex at the upstream region of the gene encoding *GATA3*, a transcription factor essential for T helper type 2 (Th2) cell differentiation. Once Th2 cells differentiate, TrxG complex associated with the TrxG component Menin binds to the whole *GATA3* gene locus, and this binding is required for the long-term maintenance of expression of *GATA3* and Th2 cytokine. Thus, STAT6-mediated displacement of PcG by the TrxG complex establishes subsequent STAT6-independent maintenance of *GATA3* expression in Th2 cells via the recruitment of the Menin–TrxG complex.

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Abbreviations used: CBP, CREB-binding protein; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; LCR, locus control region; MLL, mixed-lineage leukemia; mRNA, messenger RNA; PcG, polycomb group; PRE, polycomb response element; RNAPII, RNA polymerase II; TRE, trithorax response element; TrxG, trithorax group; TSA, trichostatin A.

Immunity against different classes of microorganisms is directed by specialized effector CD4 Th subsets, of which the best characterized are Th1, Th2, and Th17 cells (Mosmann and Coffman, 1989; Reiner and Locksley, 1995; Korn et al., 2009). IL-12–induced activation of STAT4 is required for Th1 cell differentiation, whereas IL-4–induced STAT6 activation is crucial for Th2 cell differentiation. Master transcription factors that regulate Th1/Th2/Th17 cell differentiation have been identified. T cell–specific T-box transcription factor (T-bet) appears to be a key factor for Th1 cell differentiation (Szabo et al., 2002), *GATA3* for Th2 (Zheng and Flavell, 1997; Lee et al., 2000; Zhu et al., 2010), and ROR-γt (retinoid-related orphan receptor γt) and ROR-α for Th17 (Ivanov et al., 2006; Yang et al., 2008).

GATA3 is predominantly expressed in T lymphocytes and the embryonic brain (Yamamoto et al., 1990). In peripheral CD4 T cells, the activation of STAT6 induces high-level expression of *GATA3* messenger RNA (mRNA), although the precise mechanisms underlying the STAT6-induced *GATA3* transcription remain unclear. Changes in histone modification such as H3-K9/14 acetylation and the H3-K4 methylation at the Th2 cytokine gene loci occur during Th2 cell differentiation (Löhning et al., 2002; Ansel et al., 2006; Nakayama and Yamashita, 2008), and this is mediated primarily by *GATA3* in peripheral CD4 and CD8 T cells. High-level expression of *GATA3* is required for producing large amounts of Th2 cytokines in established Th2 cells (Pai et al., 2004; Yamashita et al., 2004; Zhu et al., 2004).

The polycomb group (PcG) complex antagonizes the effect of the trithorax group (TrxG) complex (Ringrose and Paro, 2004). The TrxG complex establishes a chromatin structure permissive for transcription, in part, through the induction of methylation at histone

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H3-K4 (Milne et al., 2002; Nakamura et al., 2002), whereas the PcG complex maintains a repressive chromatin structure via the methylation of histone H3-K27 (Cao et al., 2002). The mammalian TrxG complexes contain RbBP5, Ash2L, and WDR5, which are also related to the components in the yeast Set1 complex, and a catalytic subunit that harbors the SET domain (Yokoyama et al., 2004). In contrast, PcG molecules form multimeric and heterogeneous complexes and maintain the early-determined gene expression patterns of key developmental regulators such as homeobox genes (Satijn and Otte, 1999; van Lohuizen, 1999). There are at least two types of PcG complexes, PRC1 (polycomb repressive complex 1) and PRC2 (Ringrose and Paro, 2004). Ring1B, Ring1A, Bmi1, Mel-18, M33, Pc2, Rae-28/Mph1, and Mph2 are members of a multimeric protein complex that show similarity to the PRC1 identified in *Drosophila melanogaster*. PRC2 is another PcG complex that contains Eed, Suz12, Ezh1, and Ezh2, and it possesses intrinsic methyltransferase activity for histone H3-K27 (Cao et al., 2002). In mature lymphocytes, PcG gene products appear to play several roles in the differentiation process and cell fate. Bmi1 controls the stability of GATA3 protein in developing Th2 cells (Hosokawa et al., 2006) and also memory CD4 T cell survival through the direct repression of the *Noxa* gene (Yamashita et al., 2008). Mixed-lineage leukemia (MLL) is a member of TrxG molecules and controls the maintenance of Th2 cytokine gene expression in memory Th2 cells (Yamashita et al., 2006). Menin was initially identified as a product of the MEN1 tumor suppressor gene and is known to be an essential component for DNA binding of the TrxG–MLL complex (Guru et al., 1998).

This study investigates the molecular mechanisms underlying the PcG complex– and TrxG complex–mediated regulation of *GATA3* transcription. In naive CD4 T cells, the PcG complex bound to the upstream region of the *GATA3* proximal promoter, whereas the accumulation of the Menin–TrxG complex was restricted to a part of the coding region. IL-4–mediated STAT6 activation induced the displacement of the PcG complex by the TrxG complex at the upstream region of the *GATA3* gene locus. After Th2 cell differentiation, the binding of Menin–TrxG complex was required for the maintenance of *GATA3* expression and Th2 cytokine production. This study revealed two distinct molecular processes that are critical in the regulation of *GATA3* transcription in Th2 cells: (1) IL-4/STAT6-mediated displacement of the PcG complex by the TrxG complex leading to the induction of *GATA3* transcription during Th2 cell differentiation and (2) STAT6-independent maintenance of *GATA3* expression and Th2 function via recruitment of the Menin–TrxG complex.

RESULTS

Dissociation of PcG complex and recruitment of TrxG complex to the *GATA3* gene locus during Th2 cell differentiation

The expression of *GATA3* mRNA is regulated in a tissue-specific manner. Naive CD4 T cells express a moderate level of *GATA3*, and in vitro differentiated Th2 cells and fully developed Th2 cells express higher levels of *GATA3*, whereas splenic B cells express little *GATA3* mRNA (Fig. 1 A, left). A similar tissue-specific profile in the protein expression of *GATA3* mRNA was observed (Fig. 1 A, right). Fully developed Th2 cells were established as described in Materials and methods. A schematic representation of the *GATA3* gene locus, with the location of specific primer pairs and probes for quantitative PCR used in this study, is shown in Fig. 1 B. First, the histone modification and the binding of the PcG and TrxG complexes at the *GATA3* gene locus were determined by chromatin immunoprecipitation (ChIP) assays. In B cells, Bmi1 bound to the upstream region of the proximal promoter and the region around exon 1 and exon 2 (primers #1 to #11; Fig. 1 C, dark blue line). The histone H3-K27 trimethylation (H3-K27Me3) signals were enriched at a broader but similar region (primers #1 to #15). Only weak signals for Menin and MLL binding and H3-K4Me3 were detected in B cells. However, in naive CD4 T cells, the Bmi1 signal was enriched only in the upstream region of the proximal promoter (primers #1 to #7; Fig. 1 C, turquoise line) but was low at the proximal promoter and the coding regions (primers #7 to #20). The signal pattern for H3-K27Me3 was almost exactly the same as that of Bmi1. In sharp contrast, Menin and MLL binding and H3-K4Me3 signals were enriched from the downstream region of the proximal promoter to exon 2. Fully developed Th2 cells showed strong enrichment of Menin and MLL signals from the beginning of the distal promoter to exon 5 and H3-K4Me3 signals to exon 3 (Fig. 1 C, red line). No significant Bmi1 accumulation and H3-K27Me3 were detected in fully developed Th2 cells. The real PCR product bands are shown in Fig. S1 (A and B). We detected comparable levels of histone H3 at all regions in these three types of cells (Fig. S1, C and D). The signals for H3-K4Me3 showed a similar pattern to that of Menin and MLL in all cells, whereas the H3-K27Me3 profile was similar to that of Bmi1. These results indicate that the increase in the expression of *GATA3* mRNA correlates with the dissociation of Bmi1 and the association of Menin and MLL to the *GATA3* gene locus, particularly in the upstream region of the proximal promoter (primers #3 to #6).

Next, the change of chromatin status and the PcG/TrxG binding at the *GATA3* gene locus during Th2 cell differentiation were assessed using a quantitative PCR assay system (Fig. 1 D). PcG proteins, Bmi1, EZH2, and Suz12 bound to the upstream region of *GATA3* proximal promoter in naive CD4 T cells (Fig. 1 D, left, probes (−15), (−5), and (−4)). The signal for H3-K27Me3 was also enriched in the upstream region of the proximal promoter. A progressive dissociation of Bmi1, EZH2, and Suz12 and decreased H3-K27Me3 were observed during Th2 cell differentiation. Essentially no PcG association was observed in fully developed Th2 cells (Fig. 1 D, left, probes (−15), (−5), and (−4)). In contrast, the binding of Menin, MLL, and RNA polymerase II (RNAPII) was restricted to the coding region in naive CD4 T cells (Fig. 1 D, right). H3-K4Me3 and H3-K9Ac signals were enriched around the proximal promoter (probe (0)). This accumulation of Menin,

MLL, and RNAPII was observed in developing Th2 cells accompanied by increased H3-K4Me3 and H3-K9Ac signals across the *GATA3* locus (Fig. 1 D, right, probes (−5) to (+3)). The accumulation was more prominent in fully developed Th2 cells. The total H3 levels were similar in these cells (Fig. 1 D, bottom left). Thus, the displacement of the PcG complex by the TrxG complex occurred during Th2 cell differentiation. Interestingly, the displacement was most characteristic in the region between the *GATA3* proximal and distal promoter (Fig. 1 D, probes (−5) and (−4)). A previous study showed that the reduced expression of the PcG gene caused the dissociation of PcG molecules from the target genes in the epidermal progenitors (Ezhkova et al., 2009). However, in this study, substantial levels of PcG and TrxG mRNA and protein were expressed in naive CD4 T cells, Th2 cells, and fully developed Th2 cells (Fig. S2), suggesting that the displacement is unlikely to be explained by the expression levels of these molecules.

The displacement of the PcG complex by the TrxG complex was dependent on STAT6

We assessed the role of the IL-4–STAT6 signaling pathway to help elucidate the molecular mechanisms underlying the displacement of the PcG complex by the TrxG complex at the *GATA3* gene locus. Although naive CD4 T cells from

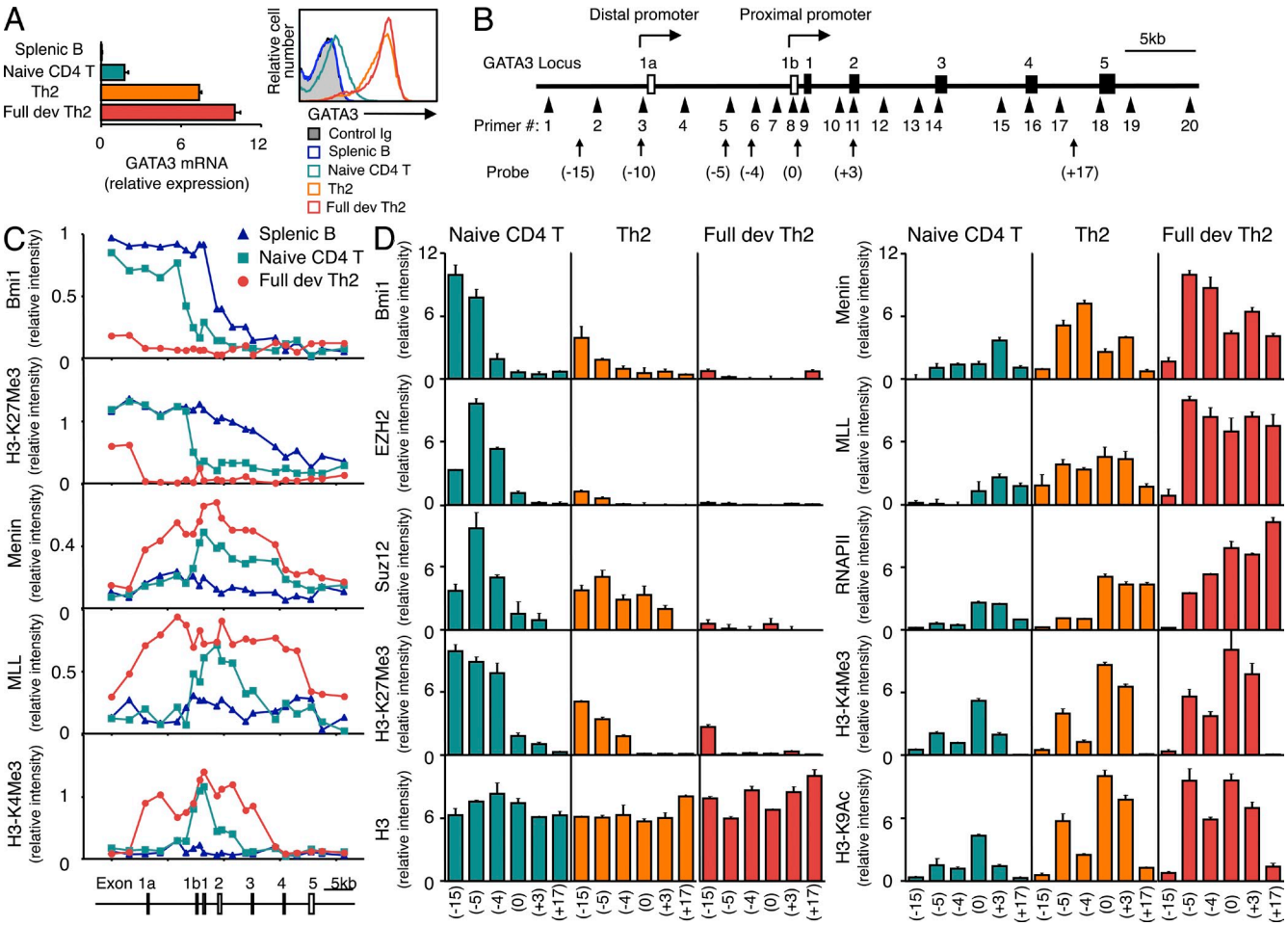

Figure 1. Changes in the histone modifications and PcG and TrxG binding to the *GATA3* locus during Th2 cell differentiation. (A) *GATA3* mRNA was measured by quantitative RT-PCR (left panel shows the relative intensity, compared with HPRT; the mean ± SD of three samples). GATA3 protein expression was determined by intracellular staining (right). Splenic B cells, naive CD4 T cells, effector Th2 cells (Th2), and fully developed Th2 cells (Full dev Th2) were used. Two independent experiments were performed with similar results. (B) Schematic representation of the mouse *GATA3* gene locus. Open rectangles indicate a noncoding exon, and closed rectangles indicate a coding exon. Closed triangles show the locations of PCR primer pairs used in the ChIP assay. Positions of TaqMan probes used for real-time quantitative PCR ChIP are indicated in relative kilobases to the *GATA3* translational start site. (C) A representative result of a ChIP assay using antibodies specific for the indicated proteins, with a series of primer pairs covering the *GATA3* locus. PCR product band intensities relative to input in each primer pair are shown. PCR product bands are shown in Fig. S1. Five independent experiments were performed with similar results. (D) Binding of the PcG complex molecule, Bmi1, EZH2, and Suz12, modifications of histone H3-K27, and total histone H3 levels at several regions around the *GATA3* gene locus were determined by ChIP assays with quantitative PCR analysis (left). Binding of the Menin–TrxG complex and RNAPII and the status of H3-K4Me3 and H3-K9Ac were also determined by quantitative ChIP assays (right). The relative intensity ([specific antibody ChIP − control Ig ChIP]/input DNA; highest signal intensity = 10; mean of three samples) is shown with SDs. Three independent experiments were performed with similar results.

STAT6-deficient mice expressed equivalent levels of *GATA3* mRNA, they failed to up-regulate *GATA3* mRNA during in vitro differentiation into Th2 cells (Fig. 2, A and B). The reduced expression of GATA3 protein in STAT6-deficient Th2 cells was confirmed by the intracellular staining of GATA3 (Fig. 2 C). WT and STAT6-deficient CD4 T cells were cultured under Th2 conditions for 5 d, and then the cells were subjected to a ChIP assay with quantitative PCR. In the case of WT and STAT6-deficient naive CD4 T cells, equivalent binding of Bmi1, Menin, and MLL and equivalent histone modifications at the upstream region and coding region of the *GATA3* gene (probes (−5) and (−4)) were observed (Fig. 2 D). In contrast, the binding of Bmi1 and the level of H3-K27Me3 at the upstream region of the proximal promoter (probes (−5) and (−4)) were reduced in WT but not in STAT6–deficient effector Th2 cells (Fig. 2 D, left). In addition, the recruitment of Menin and MLL and the induction of H3-K4Me3 and H3-K9Ac were significantly impaired in STAT6-deficient Th2 cells (Fig. 2 D, right). Although less characteristic, a similar pattern was observed at the coding region (Fig. 2 D, probe (+3)). The total H3 levels were not affected in STAT6-deficient naive and Th2 cells. The displacement of the PcG complex by the TrxG complex and corresponding histone modifications observed in Th2 cells were not observed in Th1 cells (Fig. 2 E). These results indicate that the activation of the STAT6 is crucial for the displacement of the PcG complex by the TrxG complex and that this is accompanied by the alteration in histone modification of the *GATA3* gene locus during Th2 cell differentiation.

Identification of the STAT6-binding sites within the *GATA3* gene locus

A sequence analysis identified 11 putative STAT6 consensus sites within the *GATA3* locus (from 20 kb upstream to 20 kb

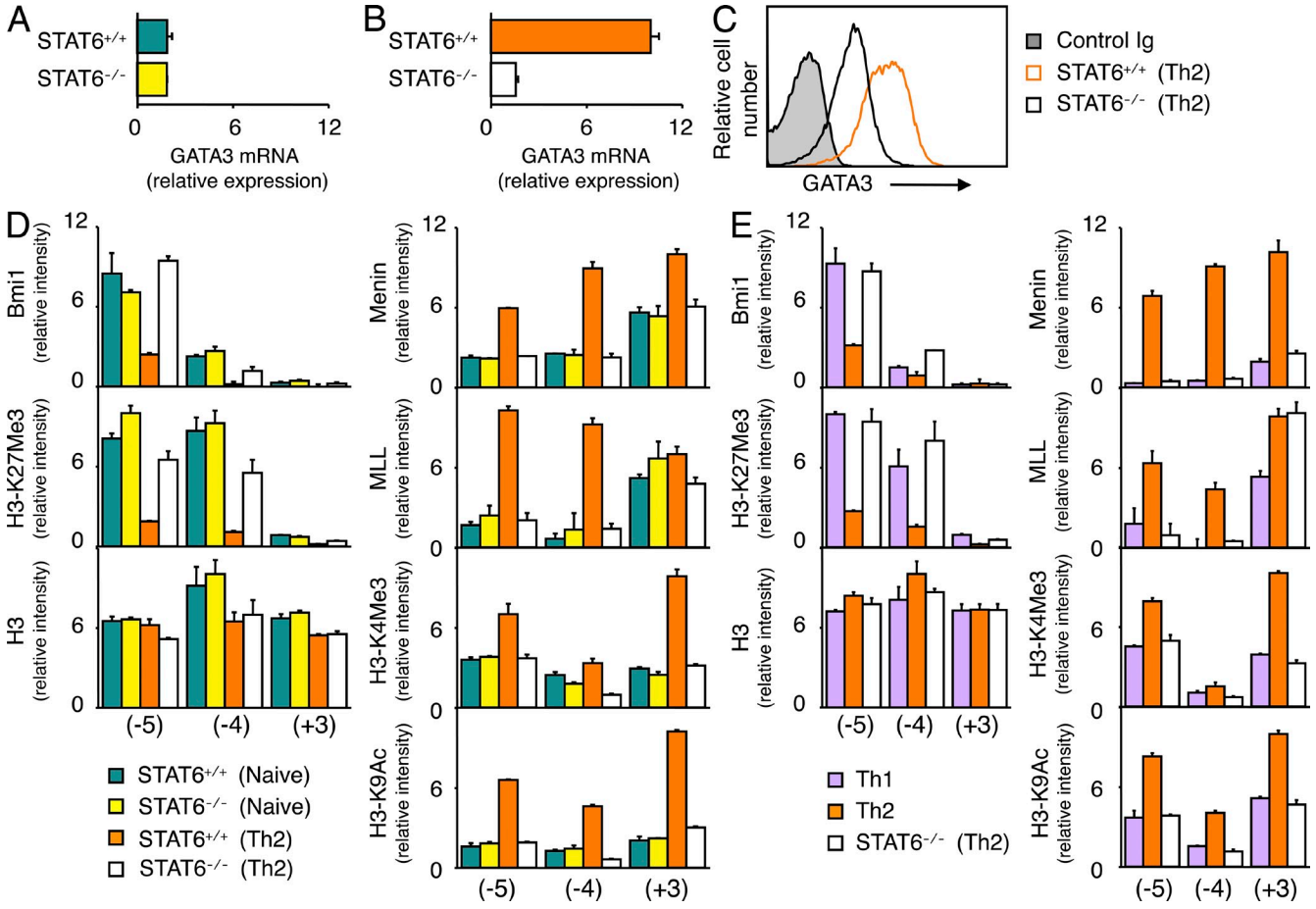

Figure 2. The displacement of the PcG by the TrxG complex at the *GATA3* gene locus was induced in a STAT6-dependent manner. (A and B) *GATA3* expression in STAT6-deficient naive CD4 T cells and Th2 cells was determined by a quantitative PCR. The relative intensity compared with HPRT (mean of three samples with SD) is shown. (C) The levels of GATA3 protein expression were determined by intracellular staining. (D) PcG and TrxG binding to the *GATA3* locus and histone modifications in STAT6-deficient naive CD4 T cells and Th2 cells were determined by a ChIP assay with a quantitative PCR analysis as described in Fig. 1 D. Positions of TaqMan probes described in Fig. 1 B are indicated in the parenthesis. The total H3 levels were included as a control. (E) PcG and TrxG binding to the *GATA3* gene locus and histone modifications in Th1, Th2, and STAT6-deficient Th2 cells were determined by a ChIP assay. Positions of TaqMan probes described in Fig. 1 B are indicated. (D and E) The relative intensity (mean of three samples) is shown with SDs. (A–E) Three independent experiments were performed with similar results.

downstream relative to the *GATA3* translational start site), and on this basis corresponding primer pairs (S1–S11) were prepared (Fig. 3 A). Splenic CD4 T cells were stimulated with IL-4 for 1 or 24 h, after which these stimulated cells were subjected to a ChIP assay with an anti-STAT6 antibody. Apparent binding was detected by the S4 and S7 primer pairs (Fig. 3 B). STAT6 binding was observed 1 h after stimulation and maintained for at least 24 h. A ChIP assay and quantitative PCR using CD4 T cells cultured under Th2 conditions for 1 h provided quantitative confirmation of the

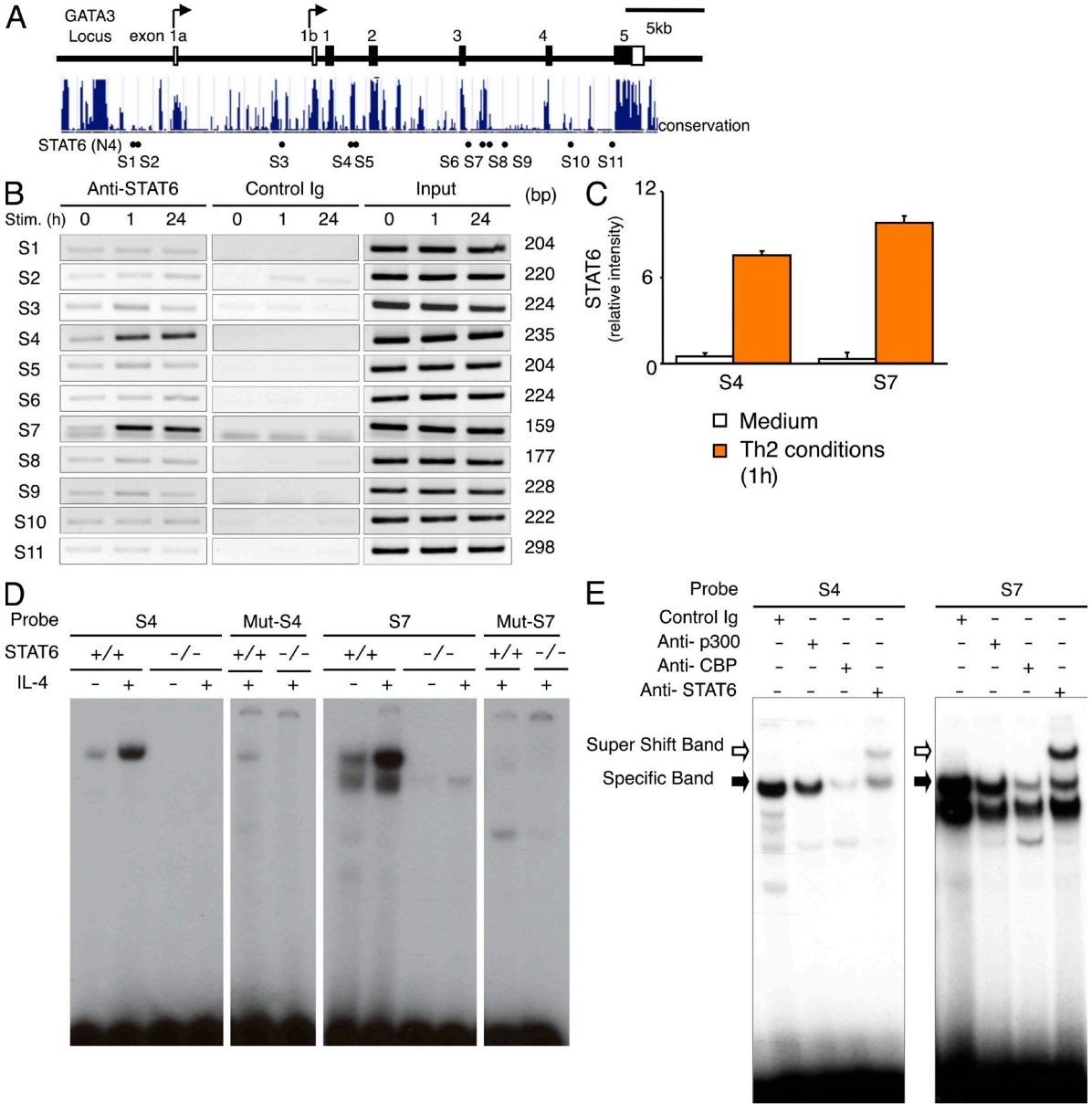

Figure 3. Identification of the binding site of STAT6 at the *GATA3* gene locus. (A) Schematic illustration of the GATA3 gene locus. 11 putative STAT-binding sites designated as S1–S11 (black dots) are indicated. The blue panel shows the conservation track obtained from the University of California, Santa Cruz Genome Browser. (B) CD4 T cells were stimulated with 100 U/ml IL-4 and immobilized anti-TCR mAb for the indicated periods. The stimulated cells were cross-linked with paraformaldehyde and then sonicated. The lysates were subjected to a ChIP assay with anti-STAT6 mAb or control Ig. The results are representative of three independent experiments. (C) CD4 T cells stimulated in medium or under Th2 cell culture conditions for 1 h were subjected to a ChIP assay. The levels of STAT6 binding at the S4 and S7 sites were determined by a quantitative PCR analysis. Three independent experiments were performed with similar results. The relative intensity (mean of three samples) is shown with SDs. (D) EMSA was performed using radiolabeled double-strand probes containing S4, Mut-S4, S7, or Mut-S7 and nuclear extracts from IL-4–stimulated WT or STAT6-deficient Th2 cells. Results are representative of two independent experiments. (E) EMSA with nuclear extracts from WT Th2 cells and radiolabeled S4 or S7 probes. Antibodies against p300, CBP, STAT6, or control mouse Ig were added to the reaction to supershift the STAT6–DNA complex. The results are representative of two independent experiments.

binding of STAT6 to the *GATA3* S4 and S7 regions (Fig. 3 C). In splenic B cells, phosphorylation and nuclear translocation of STAT6 were induced by IL-4, but STAT6 binding at the *GATA3* gene locus was not detected (unpublished data). The putative STAT6-binding sites are located at positions 1.5 kb (S4) and 10.2 kb (S7) relative to the translational start site (Fig. 3 A). To determine whether these motifs are required for the binding of STAT6, an electrophoretic mobility shift assay (EMSA) was used with nuclear extracts prepared from IL-4–stimulated T cells and incubated with radiolabeled double-stranded DNA oligonucleotide probes containing the putative STAT6-binding sites or mutated STAT6-binding sequence (probes S4, Mut-S4, S7, and Mut-S7). IL-4 treatment resulted in the formation of a nucleoprotein complex at these sites in a STAT6-dependent manner for probes S4 and S7 (Fig. 3 D) but no specific bands for the mutated oligonucleotides (Fig. 3 D, probes Mut-S4 and Mut-S7). Next, a supershift EMSA was performed using WT CD4 T cells stimulated in the presence of IL-4 (Fig. 3 E). Whereas no band shift was observed with control antibodies (Fig. 3, compare D [second through eighth lanes] with E [first through fifth lanes]), bands were supershifted by preincubation with antibodies against STAT6 (Fig. 3 E, fourth through eighth lanes), indicating that these complexes contain STAT6. Antibodies specific for HAT complex molecules, anti-p300, or anti–CREB-binding protein (CBP) mAb were also included in the supershift analysis. The intensity of the STAT6 complex band was reduced by the addition of anti-p300 mAb (second and sixth lanes) and anti-CBP mAb (Fig. 3 E, third and seventh lanes), indicating that p300 and CBP are components in the STAT6 complex. Thus, STAT6 appears to associate with the p300/CBP-containing HAT complex after IL-4 stimulation.

Trichostatin A (TSA) treatment was sufficient for the dissociation of the PcG complex from the *GATA3* locus

To further understand the role for histone modification in this process, the effect of histone deacetylase inhibitor TSA in the displacement of PcG complex by TrxG complex was examined. WT and STAT6-deficient CD4 T cells were cultured under Th2 conditions in the absence or presence of TSA for 5 d, and then the cells were subjected to a ChIP assay. The dissociation of the PcG complex (Bmi1 and EZH2) at the upstream region of the proximal promoter (probes (−5)

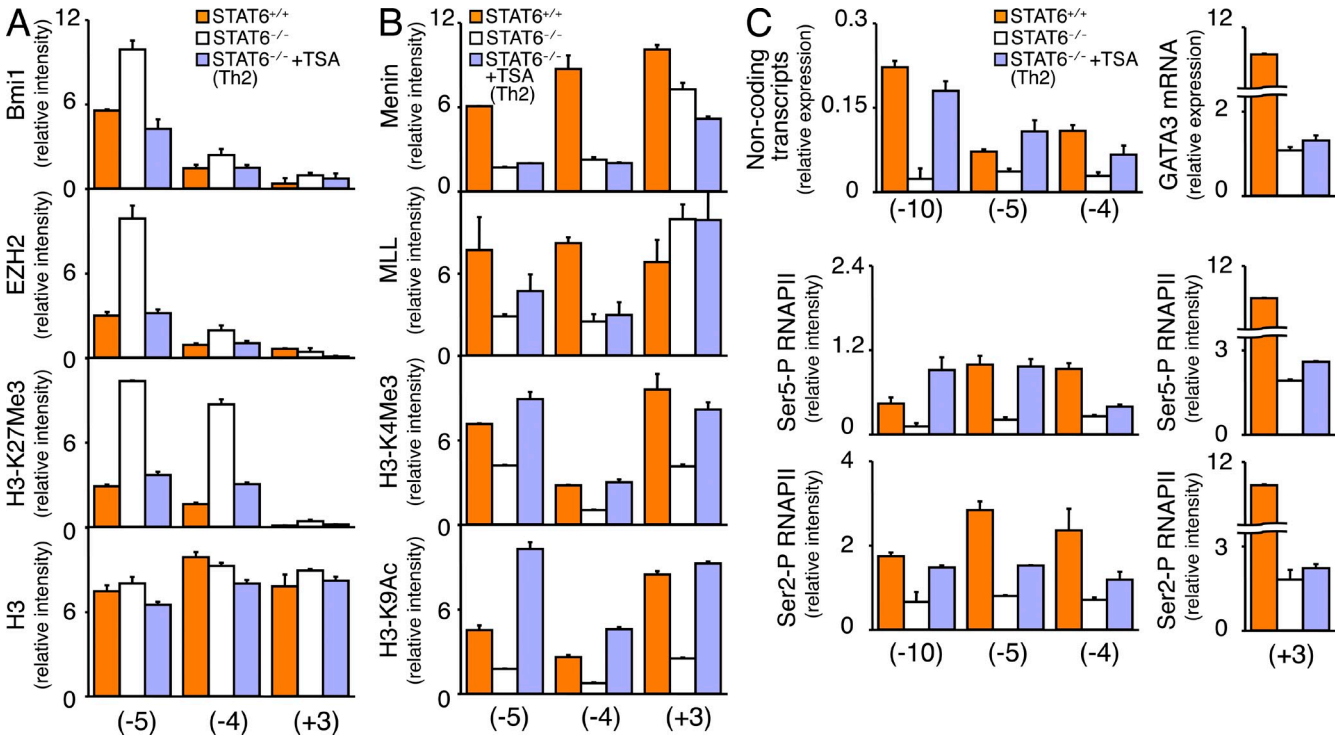

Figure 4. TSA treatment induced dissociation of PcG complex at the upstream region of the *GATA3* gene locus. Freshly prepared WT or STAT6-deficient CD4 T cells were stimulated under Th2 culture conditions, and where indicated, 10 ng/ml TSA was added on day 2. 3 d later, the cells were subjected to ChIP assays using the indicated antibodies. (A) Effects of TSA treatment on the dissociation of Bmi1 and EZH2 and the status of H3-K27Me3. The total H3 levels were included as a control. (B) Effects of TSA treatment on the recruitment of TrxG complexes (MLL and Menin) and the status of H3-K4Me3 and H3-K9Ac. (A and B) The relative intensity (mean of three samples) is shown with SDs. (C) The levels of noncoding transcripts at the *GATA3* gene locus and mature *GATA3* mRNA and HPRT in cells stimulated as in A and B were determined by quantitative RT-PCR. The relative intensity compared with HPRT (mean of three samples with SDs) is shown. Effect of TSA treatment on the recruitment of Ser5-P and Ser2-P RNAPII was determined by a ChIP assay with quantitative PCR (bottom). (A–C) Positions of TaqMan probes described in Fig. 1 B are indicated in the parenthesis. Three independent experiments were performed with similar results.

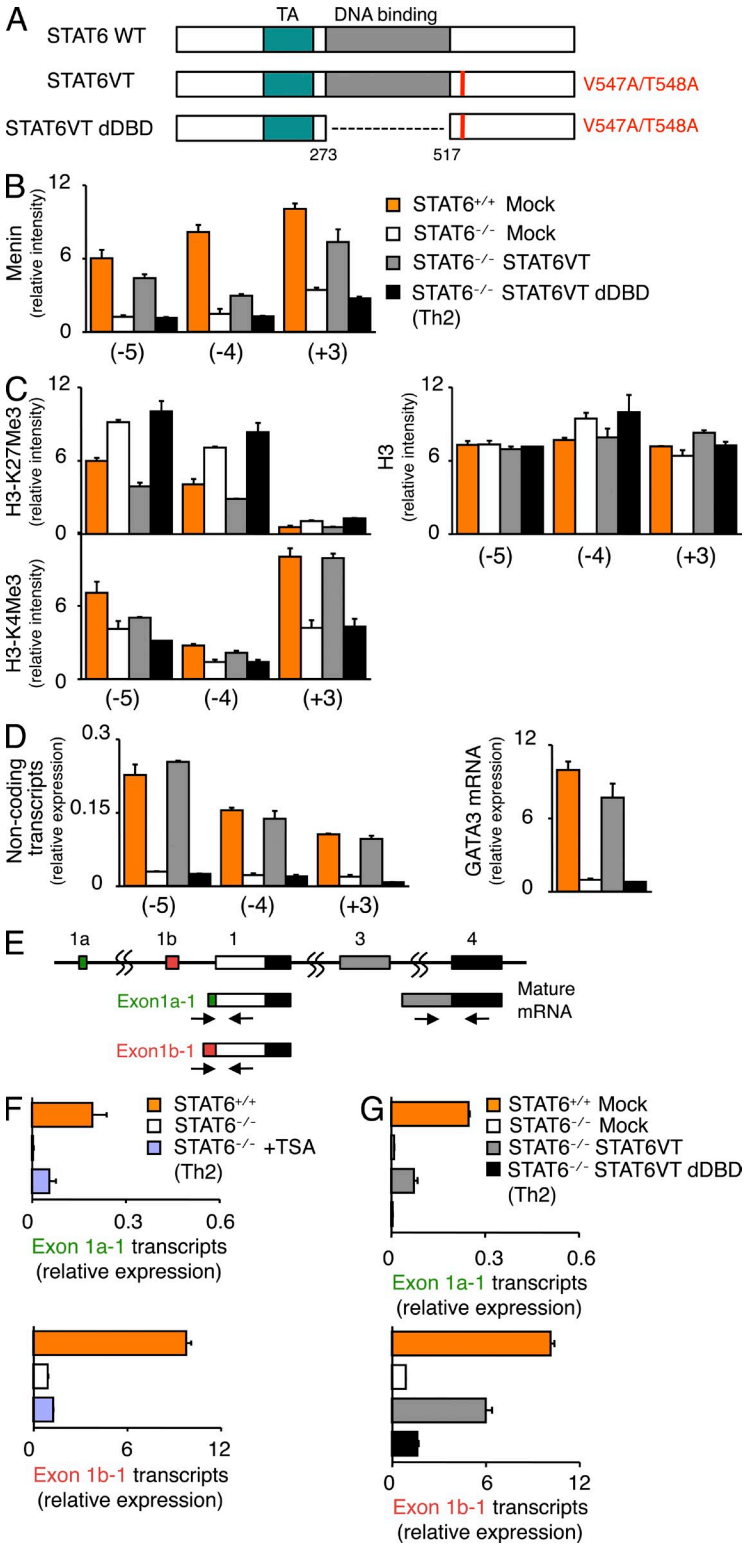

Figure 5. STAT6 activation was necessary and sufficient for recruitment of Menin and up-regulation of mature *GATA3* mRNA during Th2 cell differentiation. (A) Schematic representation of STAT6 WT, full-length STAT6VT, and DNA-binding mutant (dDBD) with location of the transactivation (TA) and DNA-binding domain indicated. STAT6VT and STAT6VT dDBD contain V547A and T548A mutations. (B–D) Freshly isolated WT or STAT6-deficient CD4 T cells were stimulated under Th2 conditions for 2 d, and then the cells were mock infected or infected with a retrovirus vector containing a STAT6VT cDNA (pMXs-STAT6VT-IRES-hNGFR) or a STAT6VT dDBD cDNA (pMXs-STAT6VT-dDBD-IRES-hNGFR). hNGFR-positive infected cells were enriched by magnetic cell sorting. (B) The levels of Menin binding to the *GATA3* gene locus were assessed by a ChIP assay. (C) Effect of STAT6VT introduction on the histone modifications was determined by a ChIP assay with quantitative PCR. (B and C) The relative intensity (mean of three samples) is shown with SDs. (D) Noncoding transcripts and mature *GATA3* mRNA were measured by quantitative PCR. (E) Schematic illustration of the *GATA3* exon 1a, exon 1b, and exon 1 and the primer sites for the amplification of the 1a (exon 1a-1)- and 1b-containing transcript (exon 1b-1). Arrows represent the position of primer pairs for PCR detection. Squiggly lines represent the break in a long section of the gene. (F and G) The levels of *GATA3* exon 1a–containing transcript and exon 1b–containing transcript were measured by exon-specific quantitative PCR analysis. (F) TSA treatment was performed as described in Fig 4. (D, F, and G) The relative expression (mean of three samples) is shown with SDs. (B–D, F, and G) Two independent experiments were performed with similar results.

and (−4)) occurred normally in STAT6-deficient Th2 cells in the presence of TSA (Fig. 4 A, top). H3-K27Me3 at the *GATA3* locus was also reduced in the presence of TSA. The total H3 levels were not influenced by TSA treatment.

In contrast to the dissociation of the PcG complex, no obvious recruitment of Menin or MLL was detected in the TSA-treated STAT6-deficient Th2 cells (Fig. 4 B, top). Interestingly, TSA treatment induced H3-K4Me3 in addition to the H3-K9Ac at the *GATA3* locus (Fig. 4 B, bottom). Next, the effect of TSA on the *GATA3* transcription was examined. The transcripts between the distal and the proximal promoter (probes (−10) to (−4)) were not induced in STAT6-deficient Th2 cells, and these transcripts were induced by the treatment with TSA (Fig. 4 C, top left). However, the TSA treatment did not induce mature *GATA3* transcript (Fig. 4 C, top right).

Recent studies indicate that the phosphorylation status at Ser2 and Ser5 is associated with the transcriptional active and poised status of the RNAPII complex (for review see Brookes and Pombo, 2009). Consequently, we performed ChIP assays to assess the binding of Ser2- and Ser5-phosphorylated RNAPII at the *GATA3* gene locus. No obvious difference in the binding pattern was observed in both WT and STAT6-deficient Th2 cells (Fig. S3). The TSA treatment resulted in the accumulation of RNAPII (both Ser2- and Ser5-phosphorylated RNAPII) between the distal and the proximal promoter (Fig. 4 C, bottom left, probes (−10) to (−4)) but not at the coding region of the proximal promoter (Fig. 4 C, bottom right, probe (+3)). No obvious

decrease but rather a slightly increased expression of Bmi1, EZH2, Menin, and MLL in the STAT6-deficient Th2 cells was seen with TSA treatment, suggesting that neither PcG complex dissociation nor TrxG complex recruitment in the TSA-treated Th2 cells is controlled by the expression levels of these molecules (Fig. S4). Thus, histone hyperacetylation itself is responsible for the dissociation of the PcG complex accompanied with down-regulation of H3-K27Me3 and noncoding transcripts at the upstream region of the proximal promoter of the *GATA3* gene; however, this alone was not sufficient for the recruitment of the TrxG complex.

The activation of STAT6 induced TrxG recruitment and up-regulation of mature *GATA3* transcript during Th2 cell differentiation

Next, we assessed whether STAT6 activation itself induced the recruitment of Menin and mature transcripts. STAT6VT, an autoactivated STAT6 molecule (Daniel et al., 2000), and a STAT6VT DNA-binding mutant (STAT6VT dDBD) molecule (Fig. 5 A) were introduced into STAT6-deficient CD4 T cells cultured under Th2 conditions. The STAT6VT- and STAT6VT dDBD–introduced cells were harvested 3 d after infection and then were subjected to a ChIP assay. STAT6VT was found to induce the recruitment of Menin between the distal and the proximal promoter and also the coding region (Fig. 5 B, probes (−5), (−4), and (+3)). STAT6VT induced the reduction of H3-K27Me3 and the up-regulation of H3-K4Me3 (Fig. 5 C). However, STAT6VT dDBD did not induce the recruitment of Menin, the reduction of H3-K27Me3, or the up-regulation of H3-K4Me3 (Fig. 5, B and C). The total H3 levels were not influenced by STAT6VT introduction (Fig. 5 C). In addition, the transcripts between the distal and the proximal promoter (probes (−10) to (−4)) were restored by the ectopic expression of STAT6VT in STAT6-deficient developing Th2 cells (Fig. 5 D, left). Furthermore, STAT6VT rescued the mature *GATA3* transcript (Fig. 5 D, right), whereas STAT6VT dDBD had no effect on *GATA3* transcription (Fig. 5 D). These results indicate that the activation of STAT6 itself induced the recruitment of Menin and the mature *GATA3* transcript during the development from naive CD4 T cells to Th2 cells.

The first alternative exons of the *GATA3* transcripts (1a and 1b) are spliced to a common exon 1, which contains the translation start site (Fig. 5 E; Asnagli et al., 2002; Scheinman and Avni, 2009). First, the levels of transcripts of both exon 1a-1 and exon 1b-1 were assessed in WT and STAT6-deficient Th2 cells in the presence of TSA (Fig. 5 F). We confirmed that the exon 1b-1 transcript was much more abundant than that of exon 1a-1 (Scheinman and Avni, 2009). The levels of both transcripts were very low in STAT6-deficient Th2 cells. Interestingly, TSA treatment partially rescued the exon 1a-1 transcript but not the exon 1b-1 transcript (Fig. 5 F). The result was similar to that seen with noncoding and mature transcripts, respectively, as shown in Fig. 4 C. Then, we assessed the effect of the ectopic expression of STAT6VT on these two transcripts and observed that rescue was partial for the exon 1a-1 transcript but was substantial in the case of the exon 1b-1 transcript (Fig. 5 G). Thus, the effect of STAT6VT on exon 1b-1 transcript was similar to that seen on *GATA3* mature transcripts, as shown in Fig. 5 D. STAT6VT dDBD showed no obvious effect on exon 1a-1 and exon 1b-1 transcripts (Fig. 5 G). These results indicate that both exon 1a-1 and exon 1b-1 transcripts are dependent on STAT6 activation and that the former is associated with noncoding transcripts of the upstream region of the *GATA3* gene and the latter is associated with the mature transcripts.

Menin was required for the maintenance of *GATA3* expression in developed Th2 cells

Menin is an essential component for DNA binding of the TrxG–MLL complex (Guru et al., 1998). A CD4-Cre–driven conditional knockout mouse for Menin (Menin-deficient mice) was used to assess the role of Menin in the TrxG recruitment in the expression of the *GATA3* gene. In Menin-deficient mice, equivalent numbers of CD4 and CD8 T cells developed in the thymus, but a 50% reduction in the number of splenic CD4 cells was observed in comparison with WT control (Fig. S5 A). The cell surface phenotype of splenic CD4 T cells was found to be within the normal range (Fig. S5 B). Proliferative responses induced with immobilized anti-TCR mAb were not impaired but somewhat slightly enhanced in Menin-deficient CD4 T cells (unpublished data). The lack of Menin mRNA in the conditional Menin-deficient Th1 and Th2 cells was confirmed (Fig. S5 C). Menin-deficient CD4 T cells cultured under Th2 conditions expressed an equivalent amount of *GATA3* transcript and protein to WT CD4 T cells (after the first cycle of stimulation; Fig. 6, A and B) and differentiated normally into effector Th2 cells (Fig. 6 D). To further probe the role of Menin, the binding of MLL and the status of H3-K4Me3 at the *GATA3* gene locus were assessed in the Menin-deficient Th2 cells. We could not detect the recruitment of MLL at the GATA3 gene locus in Menin-deficient Th2 cells (Fig. 6 C, left). Interestingly, the levels of H3-K4Me3 were decreased at the upstream region of the *GATA3* proximal promoter (probes (−5) and (−4)), whereas this was not obvious at the coding region (Fig. 6 C, left, probe (+3)). The binding of the PcG proteins, Bmi1 and EZH2, and those of H3-K27Me3 after the first cycle stimulation were reduced equivalently between the WT and Menin-deficient Th2 cells (Fig. 6 C, right). The total H3 levels were not changed in Menin-deficient Th2 cells (Fig. S6 A). These results indicate that STAT6-dependent but Menin–TrxG-independent *GATA3* transcription is induced during the first cycle of Th2 cell differentiation.

Interestingly, although the expression levels of *GATA3* transcript in WT Th2 cells was maintained even when the cells were stimulated with anti-TCR mAb in the presence of anti–IL-4 mAb (Fig. S7, second to third cycle), the expression of *GATA3* transcript in Menin-deficient Th2 cells was not maintained in the presence of anti–IL-4 mAb (after the third cycle of stimulation; Fig. 6 E). The high expression of GATA3 protein in Menin-deficient Th2 cells was also not

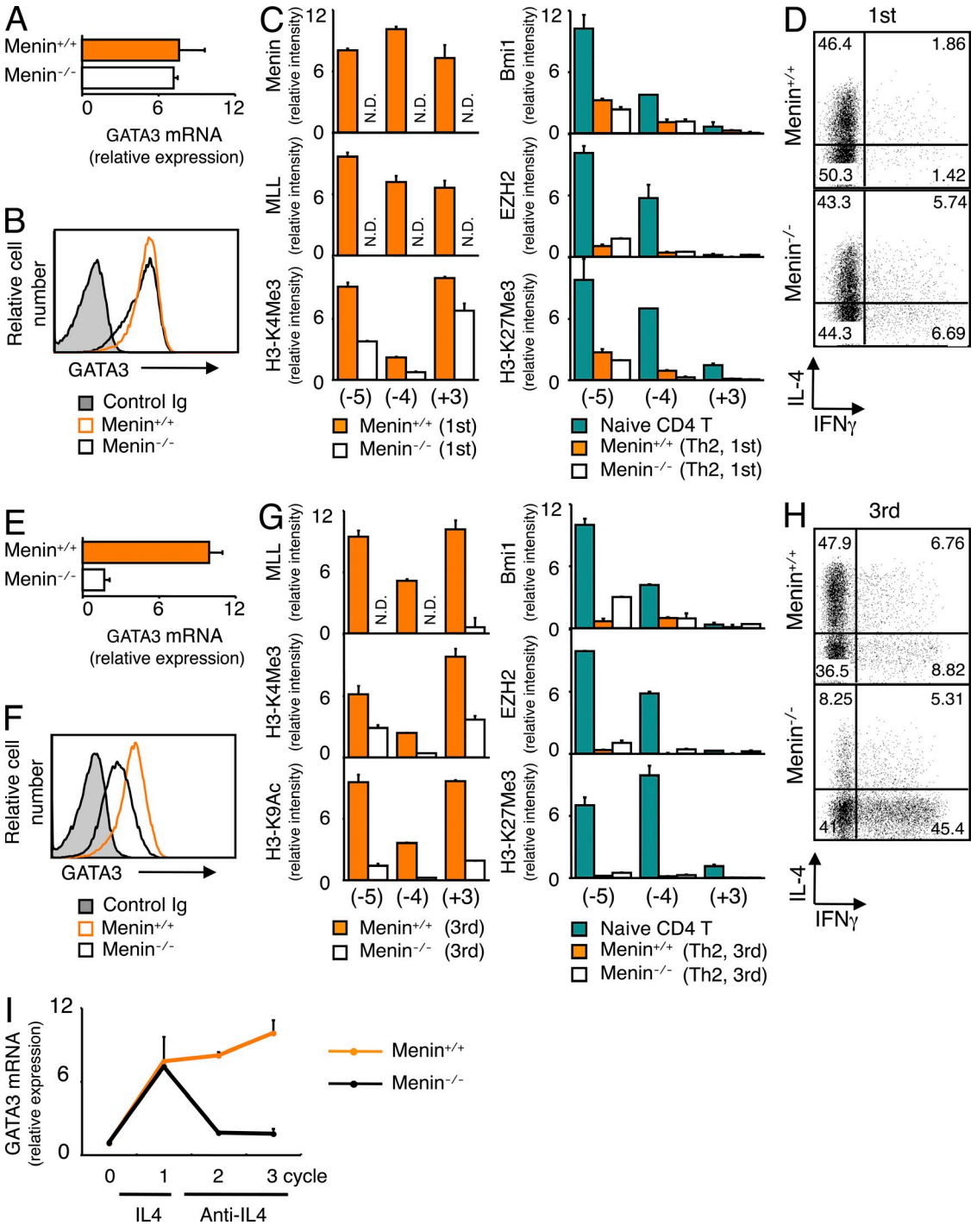

Figure 6. Menin-deficient effector Th2 cells failed to maintain the expression of *GATA3* in the absence of IL-4. (A–D) Naive CD4 T cells from WT or Menin-deficient mice were cultured under Th2 conditions for 5 d (first cycle). These Th2 cells were further cultured for 2 d in the absence of cytokines and then restimulated with anti-TCR mAb in the presence of IL-2 and anti–IL-4 mAb for an additional 5 d (second cycle). (E–H) This cycle was repeated again (third cycle). (A and E) *GATA3* mRNA was determined by quantitative RT-PCR. The relative intensity compared with HPRT (mean of three samples with the SDs) is shown. (B and F) GATA3 protein expression was determined by intracellular staining. (C and G) Histone modifications and PcG and TrxG binding to the *GATA3* locus were determined by ChIP assays with quantitative PCR analysis as described in Fig. 1 D. The relative intensity (mean of three samples) is shown with SDs. (D and H) The cultured cells were restimulated with immobilized anti-TCR mAb and monensin for 6 h, and intracellular IFN-γ and IL-4 staining profiles were examined. Representative profiles are shown with the percentages of cells in each area. (I) A time course analysis of *GATA3* mRNA expression in WT and Menin-deficient naive CD4 T cells (0 cycle), in vitro differentiated Th2 cells (first cycle), and Th2 cells cultured in the presence of anti–IL-4 (second and third cycles). The relative expression (mean of three samples) is shown with SDs. (A–I) Three independent experiments were performed with similar results.

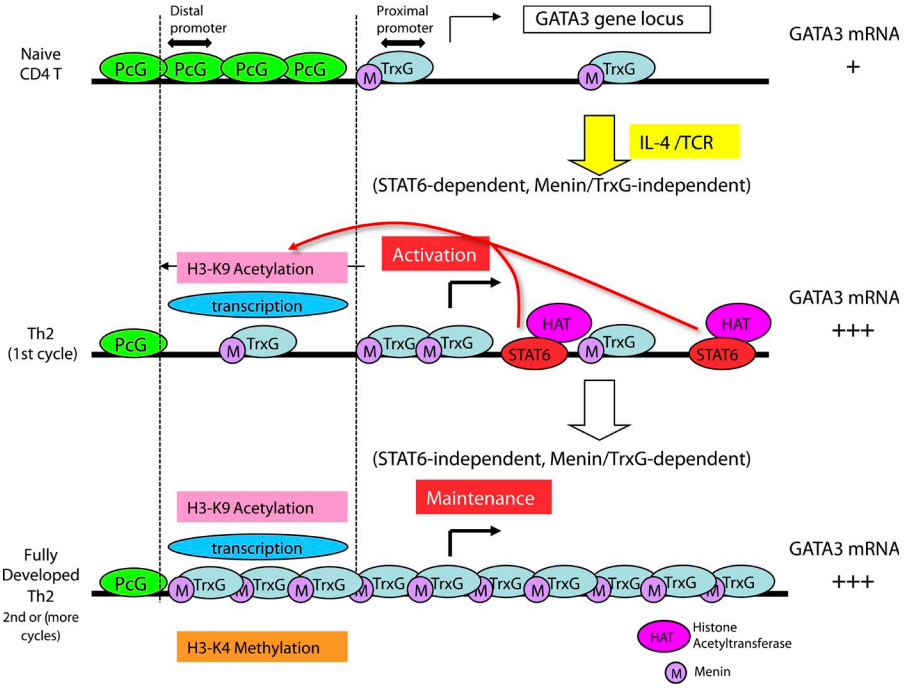

Figure 7. Schematic illustration of the transcriptional regulation of the *GATA3* gene in naive CD4 T cells, developing Th2 cells, and developed Th2 cells. In naive CD4 T cells, the PcG complex binds to the upstream region of the proximal promoter of the *GATA3* gene, and the expression of *GATA3* mRNA is moderate. After stimulation through TCR in the presence of IL-4 (Th2 culture conditions), STAT6 is activated, and STAT6 associated with the HAT complex binds to the *GATA3* gene locus. HAT-dependent histone acetylation spreads to the upstream region, resulting in the dissociation of PcG complex. A high-level *GATA3* mRNA expression is achieved in an IL-4/STAT6-dependent but Menin–TrxG-independent manner. The role of Menin–TrxG recruitment in *GATA3* transcription appears to be minimal at this stage. Once Th2 cells are developed, the Menin–TrxG complex binds to the whole *GATA3* gene region, including the upstream region of the proximal promoter. A broad range of H3-K9Ac and H3-K4Me3 is observed. The Menin–TrxG complex bound to the *GATA3* locus maintains the high expression levels of *GATA3* in the absence of IL-4. IL-4/STA6-independent but Menin–TrxG-dependent regulation of the transcription of *GATA3* is operating.

maintained (Fig. 6 F). The recruitment of MLL at the *GATA3* gene locus was not detected in Menin-deficient Th2 cells after third cycle cultivation (Fig. 6 G, left). H3-K4Me3 and H3-K9Ac levels were decreased at both the upstream and the coding regions after stimulation in the presence of anti–IL-4 mAb (Fig. 6 G, left). Interestingly, no significant increase in the binding of Bmi1 and EZH2 and in the level of H3-K27Me3 was observed even after stimulation in the presence of anti–IL-4 mAb (Fig. 6 G, right). The total H3 levels were not changed in Menin-deficient Th2 cells even after the third cycle cultivation (Fig. S6 B). These results indicate that the PcG complex did not reassociate with the *GATA3* gene locus in Th2 cells even in the absence of the binding of the Menin–TrxG complex. The ability to produce IL-4 was assessed after restimulation with anti-TCR mAb and was found to be diminished in the Menin-deficient Th2 cells after the two-cycle stimulation in the presence of anti–IL-4 mAb (Fig. 6 H). The antigenic stimulation with IL-4 for 1 wk did not restore the production of IL-4 in the Th2 cells that had received two-cycle stimulation in the presence of anti–IL-4 mAb (unpublished data), indicating that the ability to become IL-4–producing cells was lost in the absence of Menin. Menin-deficient naive CD4 T cells expressed an equivalent amount of *GATA3* transcript to WT CD4 T cells (Fig. 6 I, 0 cycle). The expression of *GATA3* transcript in Menin-deficient Th2 cells was not maintained in the presence of anti–IL-4 mAb (Fig. 6 I, second cycle). Collectively, once Th2 cells have successfully developed, they maintain the *GATA3* expression and Th2 function via the recruitment of the Menin–TrxG complex to the *GATA3* gene locus even in the absence of IL-4–mediated STAT6 activation.

DISCUSSION

This study dissects two distinct processes that are critical in the regulation of transcription of the *GATA3* gene in Th2 cells based on the binding of PcG and TrxG complexes (Fig. 7). In naive CD4 T cells, the PcG complex binds to the upstream region of the *GATA3* proximal promoter, whereas the accumulation of the Menin–TrxG complex is restricted to a part of the coding region. In developing Th2 cells, IL-4–mediated STAT6 activation induces the dissociation of the PcG complex, which is accompanied by the acetylation of H3-K9 and noncoding transcripts at the upstream region of the *GATA3* gene locus. Histone hyperacetylation is sufficient to cause the dissociation of the PcG complex and the induction of noncoding transcripts. High-level transcription of the *GATA3* gene is induced in these cells (Fig. 7, middle). The role of binding of the Menin–TrxG complex in *GATA3* transcription appears to be minimal at this stage. In fully developed Th2 cells, high-level expression of *GATA3* can be maintained in the absence of IL-4 (Fig. 7, bottom). IL-4/STAT6-independent but Menin–TrxG-dependent regulation of *GATA3* transcription is operating. A broad range binding of Menin was confirmed by a ChIP-seq assay (Fig. S8). The binding of Menin–TrxG complex to the *GATA3* gene is required for the long-term maintenance of *GATA3* expression and Th2 cytokine production. Because the supply of IL-4 is limiting in the body after the clearance of infectious organisms, this could be the mechanism by which memory Th2 cells can maintain Th2 function for a long time in vivo.

STAT6 plays several distinct roles in the regulation of *GATA3* transcription. Although STAT6 is well known to be

required for the expression of the *GATA3* gene, STAT6-binding sites that are critical for the induction of *GATA3* transcription have not been identified. We identified two STAT6-binding sites within the intron 1 and intron 3 regions of the *GATA3* gene locus at which STAT6 rapidly accumulated after IL-4 stimulation, resulting in the recruitment of the HAT complex (Fig. 3). Therefore, Th2-specific *GATA3* transcription appears to be mediated by the direct binding of STAT6. These binding sites are located several kilobases downstream from the transcriptional start site. However, this is not unusual because there are several examples in which transcription is induced by the binding of STAT family proteins at the distal locus control region (LCR; Jothi et al., 2008; Liao et al., 2008).

We also demonstrate that STAT6 binding recruited the HAT complex to the *GATA3* gene and induced the spreading of histone hyperacetylation with the resultant dissociation of the PcG complex at the upstream region of the *GATA3* gene. The two STAT6-binding sites are also located several kilobases downstream from the region where the displacement of PcG by TrxG occurs. Two likely mechanisms can be entertained. First, these STAT6-binding sites may act as cis-regulatory elements and exert enhancer activity for *GATA3* mRNA expression. At the β-globin gene locus, communication between the LCR and the active β-globin genes is generally thought to occur via a looping mechanism whereby the LCR and the β-globin genes come into direct physical contact through their interacting proteins and the intervening DNA sequences are looped out (Dean, 2006). The STAT6-binding sites may be close to the proximal or distal promoter through the high-ordered chromatin configuration, and thus, the STAT6 complex may control the dissociation of the PcG complex from the distant region of the STAT6-binding sites. Alternatively, the STAT6–HAT complex may spread from its binding sites toward the upstream region during Th2 cell differentiation and induce the dissociation of the PcG complex. This model is called the tracking or spreading model (Bondarenko et al., 2003; Dean, 2006). For example, erythroid activators NF-E2 and *GATA1* are important for the recruitment of the HAT complex to the LCR and for histone acetylation at the LCR and the distant β-globin gene (Johnson et al., 2001). The accumulation of STAT6 was observed at only two sites at the early phase of Th2 cell differentiation (Fig. 3, B and C), whereas a few days after the initial stimulation, the binding of STAT6 was broadly detected throughout the *GATA3* gene by a ChIP assay (unpublished data). Thus, we would favor the second model.

The induction of Th2-specific up-regulation of *GATA3* transcription has been suggested to result from the activation of the distal promoter of *GATA3* (Amsen et al., 2007; Fang et al., 2007). However, recent studies by other investigators have indicated that the Th2-specific increase of *GATA3* transcripts was dependent on the proximal promoter (Scheinman and Avni, 2009; Yu et al., 2009). As shown in Fig. 5 (F and G), we demonstrated that exon 1b-1 transcripts induced by the proximal promoter were much more abundant in comparison with exon 1a-1 transcript, and thus, it would appear to us that the majority of the Th2-specific increase of mature *GATA3* transcripts is dependent on the proximal promoter. In contrast, the transcript induced by the distal promoter may reflect the existence of noncoding transcripts, which may play an important role in the dissociation of the PcG complex at the *GATA3* gene (Hekimoglu and Ringrose, 2009). In fact, the induction of noncoding transcripts and that of exon 1a-1 junction-containing transcripts occurs in similar fashion. The induction of noncoding transcripts at the upstream region of the proximal promoter including the region of the distal promoter was STAT6 dependent, and the noncoding transcripts were induced by the treatment with TSA (Fig. 4 C). The transcripts from the distal promoter are spliced and form the exon 1a-1 junction (Fig. 5 E, middle). These exon 1a-1 junction-containing transcripts were also STAT6 dependent and significantly up-regulated by the treatment with TSA (Fig. 5 F). The polycomb response element (PRE) and the trithorax response element (TRE) have been well established in *Drosophila* (Schwartz and Pirrotta, 2008). Noncoding transcripts have been detected around the PRE/TRE, and they play an important role for the binding of the PcG and TrxG complexes (Schmitt et al., 2005; Hekimoglu and Ringrose, 2009). Therefore, the identification of the PRE/TRE in the region between exon 1a and exon 1b of the *GATA3* gene locus would be an interesting issue.

In summary, this study provides the first evidence indicating that the activation of the transcription factor STAT6 induces the displacement of the PcG complex by the TrxG complex, which then leads to control of the expression of the *GATA3* gene in Th2 cells. STAT6-induced histone acetylation appeared to be responsible for the dissociation of the PcG complex. The recruitment of the Menin–TrxG complex was found to be crucial for the maintenance of high *GATA3* expression in developed Th2 cells. Therefore, STAT6-dependent displacement of the PcG by the TrxG complex would allow for a permissive chromatin status of the *GATA3* gene locus in the developed Th2 cells in which a STAT6-independent regulation of transcription operates.

MATERIALS AND METHODS

Mice. C57BL/6 and BALB/c mice were purchased from CLEA. STAT6-deficient mice (Takeda et al., 1996) were provided by S. Akira (Osaka University, Suita, Osaka, Japan). Menin-deficient mice (Crabtree et al., 2003) were purchased from The Jackson Laboratory. CD4-Cre transgenic mice were purchased from Taconic. All mice used in this study were maintained under specific pathogen-free conditions and ranged from 6 to 8 wk of age. All experimental protocols using mice were approved by the Chiba University animal committee. All animal care was performed in accordance with the guidelines of Chiba University.

Reagents. Recombinant mouse IL-12 was purchased from BD, and recombinant mouse IL-4 was purchased from TOYOBO. The OVA peptide (residues 323–339; ISQAVHAAHAEINEAGR) was synthesized by BEX Corporation.

Antibodies. The antibodies used for the ChIP assay were anti–acetyl-histone H3-K9 (Millipore), anti–trimethyl-histone H3-K4 (LP Bio), anti–trimethyl-histone H3-K27 (Millipore), anti–histone H3 (Abcam),

anti–Ser5-P RNAPII (Abcam), anti–Ser2-P RNAPII (Abcam), anti-MLL (Bethyl Laboratories, Inc.), anti-Bmi1 (Santa Cruz Biotechnology, Inc.), anti-STAT6 (Santa Cruz Biotechnology, Inc.), anti-Menin (Bethyl Laboratories, Inc.), anti-Suz12 (Abcam), and anti-EZH2 (Diagenode). The antibodies used for EMSA were anti-STAT6 (Santa Cruz Biotechnology, Inc.), anti-CBP (Abcam), and anti-p300 (Millipore). The antibodies used for cytoplasmic staining were anti–IFN-γ–FITC, anti–IL-4–PE, and anti-GATA3–Alexa Fluor 647 (BD).

The generation of Th1 and Th2 cells. Th1/Th2 cells were generated as previously described (Yamashita et al., 2006). In brief, splenic CD4 T cells were stimulated with 3 μg/ml of immobilized anti–TCR-β mAb plus 1 μg/ml anti-CD28 mAb under the Th1 or Th2 culture conditions for 5 d in vitro. Th1 conditions were as follows: 25 U/ml IL-2, 10 U/ml IL-12, and anti–IL-4 mAb. Th2 conditions were as follows: 25 U/ml IL-2 and 100 U/ml IL-4. These cells were used as either Th1 or Th2 cells.

Establishment of fully developed Th2 cells. Splenic CD4 T cells from DO11.10 OVA-specific TCR transgenic mice were stimulated with an OVA peptide (1 μM Loh15) plus APC under Th2 culture conditions for 5 d in vitro. The Th2 cells were further cultured in vitro for 2 d in the absence of any exogenous cytokines. The cultured CD4 T cells were then restimulated with OVA peptide (1 μM Loh15) plus APC with IL-2 and anti–IL-4 mAb for 5 d. This cycle was then repeated more than three times.

Quantitative RT-PCR. Total RNA was isolated using the TRIZOL reagent (Invitrogen). cDNA was synthesized using oligo (dT) primer and Superscript II RT (Invitrogen). Quantitative RT-PCR was performed as described previously using a sequence detection system (ABI Prism 7500; Applied Biosystems; Yamashita et al., 2006). The primers and TaqMan probes for the detection of *GATA3*, Bmi1, EZH2, Menin, MLL, and HPRT were purchased from Applied Biosystems and Roche, respectively. The specific primers and Roche Universal probes used are described in Table S1. The expression was normalized by the HPRT signal.

ChIP assay. ChIP was performed using ChIP assay kits (Millipore) as previously described (Yamashita et al., 2006). Quantitative representations of the results are shown as relative band intensities measured by a densitometer (AE6905H [ATTO] and CS Analyzer version 2.08b). The specific primers used are described in Table S1. Real-time quantitative PCR analysis was performed on an ABI Prism 7500 real time PCR machine with TaqMan probes and primers (sequences available in Table S1). To calculate the enrichment of each protein to a particular target DNA, values obtained (via the standard curve method) for each target were divided by the amount of the corresponding target in the input fraction. Enrichments obtained from mock immunoprecipitations performed in parallel with normal IgG were then subtracted from the enrichment values obtained with specific antibodies ([specific antibody ChIP − control Ig ChIP]/input DNA). All the enrichments are expressed as a function of the highest enrichment obtained on the locus (set to 10; Demers et al., 2007).

EMSA. EMSAs were performed using a gel shift assay system (Promega) as described previously (Kimura et al., 2005). In brief, the nuclear extracts were incubated at room temperature with a ^{32}P-labeled, double-stranded oligonucleotide in DNA-binding buffer. In some experiments, the nuclear extracts were preincubated at 4°C with specific antibodies. Electrophoresis was conducted on 4% native polyacrylamide gel (acrylamide/bisacrylamide ratio 29:0.8 in 0.5× Tris-borate-EDTA), and the radioactivity was visualized by autoradiography. The oligonucleotides used in this experiment were normal probe S4, 5′-CTTGGCGTTCAAGAGAATTCTCAA-3′; mutant probe S4, 5′-CTTGGCGTTC***GGTTT***AATTCTCAA-3′; normal probe S7, 5′-AGCCAACTTCCTAGGAAAAAGCTG-3′; and mutant probe S7, 5′-AGCCAACTTC***AGTTT***AAAAAGCTG-3′. The STAT6 consensus motif is underlined, and the mutated nucleic acids are shown in italicized and bold characters.

TSA treatment. STAT6-deficient splenic CD4 cells were cultured under Th2 conditions, and 10 nM TSA (Sigma-Aldrich) was added in the culture on day 2. After another 3-d culture, CD4 T cells were collected for the ChIP assay and RT-PCR.

Retroviral vectors and infection. The pMX-IRES-hNGFR plasmid was generated as previously described (Shinnakasu et al., 2006). Retrovirus vectors containing STAT6VT cDNA (pMXs-STAT6VT-IRES-hNGFR; Daniel et al., 2000) or STAT6VT dDBD cDNA (pMXs-STAT6VT-dDBD-IRES-hNGFR) were used. The infected cells were enriched by magnetic cell sorting with anti-hNGFR (clone C40-1457; BD) and were subjected to a ChIP assay.

A resting and restimulation culture system. Naive CD4 T cells from Menin-deficient mice were cultured under Th2 conditions for 5 d. The Th2 cells were further cultured for 2 d in the absence of cytokines added and then restimulated with anti-TCR mAb in the presence of IL-2 and anti–IL-4 mAb for an additional 5 d. This cycle was then repeated. PcG/TrxG binding and histone modifications were detected by ChIP assays, and the *GATA3* expression was determined using quantitative RT-PCR at the end of the each cycle.

Immunoblot analysis. Cytoplasmic extracts and nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagent (Thermo Fisher Scientific). The antibodies used for the immunoblot analysis were anti–histone H3 (Abcam), anti-Erk1 (Santa Cruz Biotechnology, Inc.), anti-MLL (Bethyl Laboratories, Inc.), anti-Bmi1 (Santa Cruz Biotechnology, Inc.), anti-Menin (Bethyl Laboratories, Inc.), and anti-EZH2 (Diagenode).

ChIP-seq and Illumina sequencing. For ChIP-seq analysis, immunoprecipitate and input samples were prepared using ChIP-Seq Sample Prep kit (Illumina, Inc.). Adaptor-ligated DNA fragments were size-fractionated by 12% acrylamide gel, and the 150–250-bp fraction was recovered. DNA thus obtained was amplified by 18 cycles of PCR. 1 ng of the DNA was used for the sequencing reaction of the GAIIx (Illumina, Inc.) according to the manufacturer's instructions. 150,000–250,000 clusters were generated per tile, and 36 cycles of the sequencing reactions were performed. Short-read sequences were aligned to the mouse genome sequences (mm9 as from the University of California, Santa Cruz Genome Browser) using the Eland program. Sequences allowing no more than two mismatches per sequence were used for the analysis.

Online supplemental material. Fig. S1 shows the real PCR product bands of the results shown in Fig. 1 C. Fig. S2 shows expression of Bmi1, EZH2, Menin, and MLL in naive CD4 T cells, Th2 cells, and fully developed Th2 cells. Fig. S3 shows the binding pattern of phosphorylated RNAPII at the *GATA3* gene locus. Fig. S4 shows that expression of Bmi1, EZH2, Menin, and MLL was not affected by TSA treatment in the STAT6-deficient Th2 cells. Fig. S5 shows phenotypic characterization of peripheral CD4 T cells in Menin-deficient mice. Fig. S6 shows the levels of total H3 at the *GATA3* gene locus in Menin-deficient Th2 cells. Fig. S7 shows IL-4/STAT6-independent maintenance of *GATA3* expression in developed Th2 cells. Fig. S8 shows binding of Menin protein to specific regions around the *GATA3* gene locus. Table S1 shows primers and probes used for ChIP and RT-PCR. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20100760/DC1.

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